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ERRATUM.

Withdrawal of Claim that Azo Blue Causes Congenital Malformations.

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In a recent paper(1) in this Journal I presented data indicating that injection of a solution of the diazo dye azo blue into pregnant rats caused congenital malformations in the offspring. The experiment could not be repeated in the laboratory of Professor H. Tuchmann-Duplessis, University of Paris, who was gracious enough to inform me of this fact immediately. As a consequence, I returned the sample of my dye to the supplier for analysis. He finds that the substance purchased by me as azo blue was in reality the closely related dye trypan blue. This makes it necessary that I withdraw the claim that azo blue causes malformations as described in the published paper. The results attributed to the action of azo blue become valid if ascribed, instead, to trypan blue. All other observations in the same paper stand as presented.

An investigation into the manner in which the mistake was originally made revealed that the lot of dye in question was manufactured in 1941 as trypan blue. It was divided into two batches and placed in separate containers, each bearing the same lot number. For reasons not known, one of these containers was mislabelled azo blue while the other was correctly labelled trypan blue, although both retained the correct lot number. The company which manufactured the dye and originally mislabelled it is no longer in business. Needless to say, the company that has recently distributed the dye has made every effort to recall all samples of the erroneously labelled product, I am grateful to Professor Tuchmann-Duplessis for informing me promptly of his negative results, and to the editor of the Proceedings for affording this opportunity to correct the error.

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1. Wilson, J. G., Proc. Soc. Exp. Biol. and Med., 1954, v85, 319-322.

Serum Neutralization of Distemper Virus in Chick Embryos.* (21266)

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Virus-neutralization tests in embryonated hen's eggs have been employed by several investigators to provide evidence for the presence of distemper neutralizing antibodies (1-5). The successful cultivation of canine distemper virus in chick embryos(1,2,6) made available a new and valuable tool in distemper research and provided a cheap and adequate means of determining the presence of distemper antibody in serum.

The purpose of this paper is to present data showing that a definite correlation exists between the results of virus-neutralization tests in chick embryos and ferret protection tests, and that some samples of human serum and gamma globulin have distemper neutralizing substances which are as abundant as those known to exist in immune ferret serum.

Materials and methods. The Lederle strain of egg-adapted distemper virus(1) was employed in this study. The virus was maintained by successive serial passages on the chorioallantoic (C-A) membrane of embryonated hen's eggs. When inoculated on the C-A membranes of 8-9 day old embryos, the virus causes a marked thickening of the membrane with numerous macroscopic light-gray lesions. These lesions tend to coalesce and form large inflammatory and necrotic areas. Photomicrographs of infected and normal membranes are shown in Fig. 1. When no virus is present or when virus is neutralized by

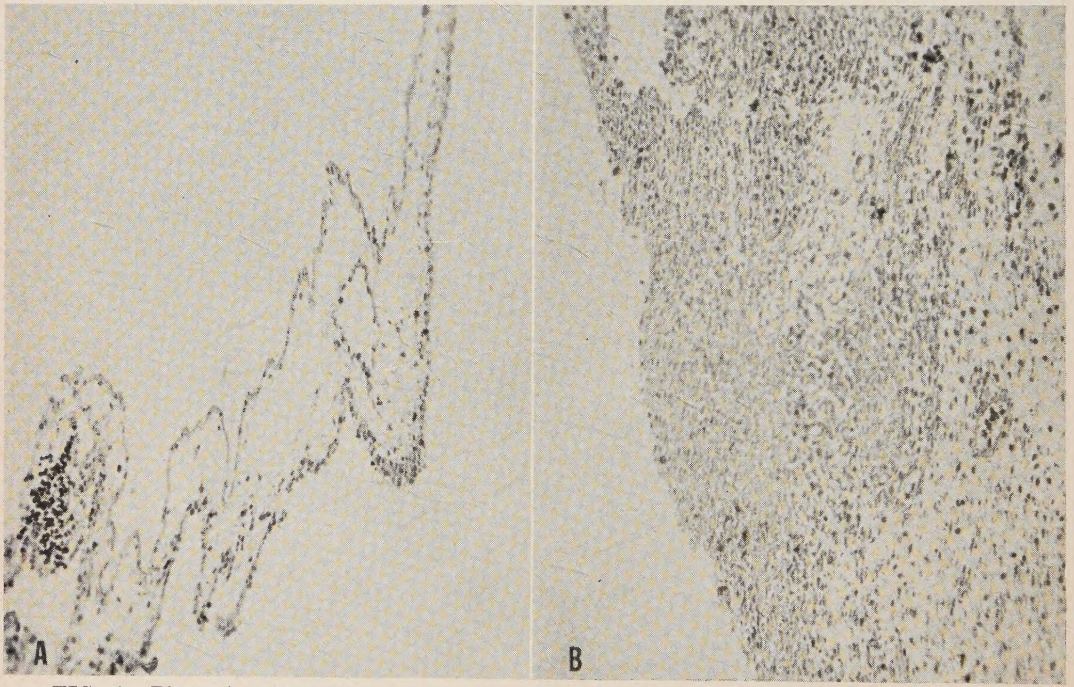

FIG. 1. Photomicrographs: A. Normal C-A membrane (×240). B. Inflammatory and necrotic membrane, 7 days after inoculation with egg-adapted distemper virus (×240).

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* Supported in part by a research grant from the National Institutes of Health, Public Health Service, and the Mead Johnson Co.

TABLE I. Results of Quantitative Serum-Virus Neutralization Tests in Eggs, Employing 20 ID$_{50}$ Egg-Adapted Distemper Virus and Samples of Ferret Serum.

Ferret No.	Serum dilutions					Results of challenge with 100 MLD ferret virus
	1:40	1:80	1:160	1:320	1:640	
N*74	2/4†	5/5	5/5			Died
N 76	—	4/5	5/5			"
N 90	3/3	4/4	4/4			"
N 91	4/5	4/5	4/5			"
I 72			0/4	0/4	—	Survived
I 85			0/5	0/5	2/4	"
I 86			0/4	0/4	0/5	"
I 88			0/5	0/4	1/5	"

* N = Normal; I = Immune.

† Numerator indicates No. of eggs showing lesions. Denominator indicates total No. of eggs.

specific antiserum, the C-A membrane remains typically thin and transparent with no apparent lesion.

Immune sera were collected from ferrets which had been immunized with the Lederle egg-adapted strain and subsequently challenged with virulent ferret-adapted distemper virus. The hyperimmune canine distemper antiserum, and the virulent ferret distemper virus used for challenge exposure of ferrets, were obtained from Lederle Laboratories through the courtesy of Drs. Herald Cox and Victor Cabasso.

Two methods were employed in the virus-neutralization tests: a) serum-dilution method and b) virus-dilution method. In the serum-dilution method, the serum was diluted in serial 2-fold dilutions. Equal volumes of diluted serum and 20 ID$_{50}$ of egg-adapted distemper virus were mixed, incubated at 37°C for 2 hours and placed in a refrigerator for an additional one-half hour. The serum-virus mixture was inoculated in a dose of 0.3 ml per egg; groups of 5-6 eggs per dilution were employed. The C-A membranes were observed following the incubation of the eggs for 6-7 days.

In the virus-dilution method, the virus was serially diluted in 2-fold dilutions. Equal amounts of diluted virus and 1:10 dilution of serum were mixed, incubated and inoculated on the C-A membranes. All samples of serum were inactivated at 56°C for one-half hour before being used in the neutralization test.

Results. For evaluation of the neutralizing capacities, sera from immune ferrets were compared with sera from ferrets with a history of no known exposure to distemper virus. The results from a typical experiment are shown in Table I. It can be seen that there were no detectable antibodies in 1:40 dilutions of the sera from normal ferrets. That these animals had no protective antibody can be further shown by the results of challenge exposure with approximately 100 MLD of ferret virus. All the ferrets showed typical symptoms of distemper and were dead by the 16th day.

On the other hand, immune ferret sera showed demonstrable antibody when diluted to 1:640. All these animals survived the challenge exposure of 100 MLD of ferret virus.

Table II shows the results from one typical quantitative neutralization experiment employing 20 ID$_{50}$ of egg-adapted distemper

TABLE II. Results of Quantitative Serum-Virus Neutralization Tests in Eggs, Employing 20 ID$_{50}$ Egg-Adapted Distemper Virus and Various Samples of Serum.

Serum samples	Serum dilutions						PD$_{50}$ of serum†
	1:80	1:160	1:320	1:640	1:1280	1:2560	
Canine hyperimmune	0/5*	0/5	0/5	0/4	2/5	5/5	1:1436
Human gamma globulin	0/6	0/4	0/6	4/6	5/5		1:537
Immune ferret	0/5	0/5	0/5	2/4			1:640
Normal ferret	5/5	5/5	5/5				<1:20

* Numerator indicates No. of eggs showing lesions. Denominator indicates total No. of eggs.

† Expressed as dilution of serum which protected 50% of embryos from infection.

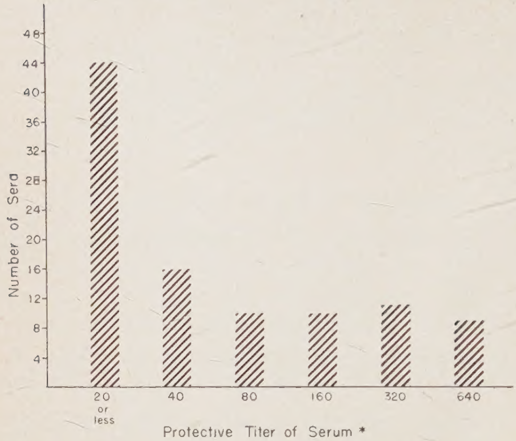

FIG. 2. Results of neutralization tests in eggs, employing egg-adapted distemper virus and 100 samples of human serum.

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* Expressed as reciprocal of dilution of serum which protected 50% of embryos from infection.

virus and various samples of serum. The resulting titers were expressed as the dilution of serum calculated to protect 50% of the embryos from infection (PD_{50}). The results indicate that human gamma globulin† has a protective titer equal to that found in immune ferret serum.

A few samples of serum from adult human beings have also shown protective titers (1:640) as high as those known to exist in immune ferret serum. The data on the first 100 samples of human serum which were tested for protective titers are presented in Fig. 2. Forty-four per cent of the sera showed some protective substance at 1:20 dilution or less. Protective titers of 1:320 dilution or greater were found in 20% of the tested samples and 9% of the sera showed titers at 1:640 dilution. Tests on samples of serum from 12 normal premature infants have shown some protective substances at 1:20 dilution of serum but not at 1:40 dilution.‡

The results of neutralization tests employing the virus-dilution technic are shown in Tables III and IV. The control materials represent sera from respective ferrets before the animals were immunized. The titer of the virus is expressed as the dilution calculated to infect 50% of the embryos. The resulting neutralization index was determined by obtaining the difference between the log titer of the virus found with the immune serum and that obtained with the control or normal serum. The results in Tables III and IV show that the neutralization indexes of the immune sera fell between 10-16. If the average for log ID_{50} titer of virus with normal serum is taken as 3.0, the neutralization index of the human gamma series will be as great or greater than that shown with the immune ferret serum. These results would again indicate that human gamma globulin has a protective titer equal to that found in specific immune sera.

Discussion. The retention of the antigenicity of the canine distemper virus even after continued passage in embryonated hen's eggs (1,2,6) has led to its use as a tool for neutralization studies. The data presented in this report indicate that the results of neutralization tests employing the egg-adapted distemper virus show a definite correlation with ferret protection tests. Animals with high titers of distemper antibodies as shown by the neutralization studies will survive the challenge exposure of virulent virus. Moreover, animals without demonstrable neutralizing antibody succumb to the challenge exposure. Therefore, the neutralization test is believed to offer a suitable method for the estimation of distemper antibody levels in serum.

The nature of the endpoint employed for the neutralization test is the presence or absence of gross macroscopic lesions on the C-A membrane. Since a constant lesion is produced by a fixed dose of virus, it is probably preferable to employ the serum-dilution technic. However, the present study also indicates that the virus-dilution technic may be employed with satisfactory results. The levels of neutralization indexes obtained with the immune sera in the present study might be explained by the relatively low level of infective titer of the avianized distemper virus in embryonated eggs. The virus was prepared in 2-fold dilutions rather than in $\log_{10}$ increments.

The presence in high titer of distemper an-

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† E. R. Squibb and Sons, N. Y.

‡ Dr. Robert Chinnock and Dr. Phyllis Wright kindly furnished these samples of serum.

TABLE III. A Representative Example of a Neutralization Test Employing the Virus Dilution Technic.

Serum samples	Virus dilutions 1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	ID_{50} titer log of virus dilution	Neutralization index
N*F89	—	4/4†	4/4	3/5	3/5	0/5	0/5	2.8	
I F89	2/4	2/5	0/4	0/4	0/4	0/5	—	1.8	10

* N = Normal; I = Immune.

† Numerator indicates No. of eggs showing lesions. Denominator indicates total No. of eggs.

tibody in human gamma globulin and some samples of human serum was also demonstrated. The significance of these findings can not be determined at the present time. A possible direct relationship between canine distemper and a respiratory disease of humans has been suggested by Adams(4).

Summary. Virus-neutralization tests were carried out in embryonated hen's eggs employing the egg-adapted distemper virus and various samples of serum. Ferrets whose sera contained no demonstrable antibody at 1:40 dilution all succumbed to 100 MLD of ferret distemper virus, whereas ferrets showing antibody titer to 1:640 dilution of serum all survived the same challenge dose. The data presented demonstrate that the results of serum-neutralization tests in chick embryos have a definite correlation with ferret protection tests. Human gamma globulin and some samples of human serum have neutralizing substances in titers as high as those known to exist in immune ferret serum. On the other hand, samples of serum from normal premature infants did not show any neutralizing substances in 1:40 dilution of the serum.

TABLE IV. Results of Serum-Virus Neutralization Tests in Eggs, Employing the Virus Dilution Technic.

Serum samples	Log ID_{50} titer of virus: Control serum	Immune serum	Neutralization index
F85	3.2	2.1	13
F86	3.0	1.9	13
F89	2.8	1.8	10
F88	3.0	1.8	16
Human gamma globulin		1.4	
Canine hyperimmune serum		.7	

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3. Cabasso, J. V., and Cox, H. R., *Cornell Vet.*, 1952, v42, 96.

4. Adams, J. M., *Pediatrics*, 1953, v11, 15.

5. Imagawa, D. T., Yoshimori, M., and Adams, J. M., *Bact. Proc.*, 1953, p50.

6. Haig, D. A., *Onderstepoort J. Vet. Sci. and Animal Industry*, 1948, v23, 149.

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Effect of Dicumarol on Localized Shwartzman Reaction.* (21267)

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(Introduced by M. A. Andersch.)

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There have been numerous theories as to the pathogenesis of the Shwartzman reaction. One theory is that it is caused by excessive concentration of toxin in the prepared site after the intravenous or provocative injection due to a localizing effect of the inflammatory reaction. This would appear to be contradicted by the fact that local injection of large amounts of toxin into the skin does not produce a dermal Shwartzman reaction. Another theory is based on the fact that skin tissues which have been prepared for the dermal Shwartzman are metabolically abnormal in that they produce large amounts of lactic acid aerobically(1). An additional finding is that rabbits given intravenous toxin become highly susceptible to the necrotizing effect of a proteolytic enzyme injected intradermally. On the basis of the preceding facts it was suggested that the local Shwartzman might be due to activation of a tissue protease in the skin. Stetson(3) suggested that the Shwartzman reaction was due to clumping of platelets and white cells occluding the vessels in the affected area and leading to hemorrhagic necrosis. The work of Good and Thomas(2) supports this theory. They found that heparin in doses large enough to prolong the clotting to 60 minutes or longer would prevent both the local and generalized Shwartzman reactions. Heparin, in addition to its anticoagulant action has effects on the proteins and lipids of the blood(4). However, Good and Thomas felt that since the action of heparin in preventing the Shwartzman reaction seemed to be closely correlated with its alteration of the clotting mechanism, it was most likely mediated through its effect on blood coagulation.

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* Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for opinions expressed or conclusions drawn by authors.

The present study was undertaken to elucidate further the action of anticoagulants on the Shwartzman reaction. It is generally agreed that heparin inhibits both the conversion of prothrombin to thrombin and that of fibrinogen to fibrin(5). Dicumarol inhibits the clotting mechanism by depressing the levels of prothrombin and factor VII in the blood. It was felt that by using dicumarol in place of heparin, it would be possible to see if the Shwartzman reaction could be inhibited by anticoagulants whose mechanism of action was different from that of heparin.

Materials and methods. Meningococcus toxin was prepared from meningococcus strain 44B,† cultured by heavy seeding on the surface of Mueller-Hinton starch agar in Kolle flasks and grown for 48 hours in candle jars. The growth in each bottle was washed off with approximately 4 cc of physiological saline. The resultant suspensions were then centrifuged for 30 minutes and the supernatant fluid was stored for 48 hours at 4°C. At the end of this time the material was cultured and was used only if the cultures were negative. The dermal Shwartzman reaction was produced by giving .25 cc of a 1:2 dilution of toxin intradermally. This was followed in 24 hours by an intravenous injection of 2 cc of a 1:30 dilution of toxin. The dermal site was then inspected for the presence or absence of hemorrhagic necrosis 5 hours after the provocative injection. The rabbits used were 1-2 kg hybrid albino rabbits, divided into 2 groups. One group served as controls and the other received dicumarol. The treated group received 50 mg of dicumarol daily till the prothrombin time was 40 seconds or over. They were then given the preparatory injection of toxin and 24 hours later, after the prothrombin time was again checked, they were given the provocative injection. They were then given the preparatory injection of toxin and 24 hours later, after the prothrombin time was again checked, they were given the provocative injection. It was found impractical to try to prolong the prothrombin time much over 40 seconds because of the hemorrhagic complications encountered.

TABLE I. Control Group, 10 Rabbits.

Prothrombin time on 5/5, sec.	Result
10	Negative
12	Hem. necrosis
11	*Idem*
11	”
10	”
10	”
11	Negative
9	Hem. necrosis
9	*Idem*
9	”

On 5/5 all rabbits were given 0.25 cc of 1:2 dilution of meningococcus toxin intradermally. On 5/6, 24 hr later, 2 cc of a 1:30 dilution of toxin was given intravenously.

Results for 16 rabbits are in Tables I and II.

Discussion. It may be seen from these results that there was no significant difference between the treated and control groups, 5 of

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† Obtained through the courtesy of Dr. Gregory Shwartzman, Mt. Sinai Hospital, New York City.

TABLE II. Treated Group, 6 Rabbits.

Pro time value with date obtained	Sec.	Date & time preparatory inj. given	Pro time value with date just before provocative inj.	Sec.	Date provocative inj. given	Results
5/17 3 P.M.	40	5/17 6 P.M.	5/18 5 P.M.	51	5/18 6 P.M.	Hem. necrosis
Idem	55	*Idem*	*Idem*	105	*Idem*	*Idem*
"	50	"	"	90	"	"
"	45	"	"	50	"	"
"	44	"	"	40	"	Negative
5/18 10:30 A.M.	60	5/18 1 P.M.	5/19 1 P.M.	60	5/19 1:45 P.M.	Hem. necrosis

6 of the treated group and 8 of 10 of the controls showing hemorrhagic necrosis.

It would seem, therefore, that the effect of heparin in inhibiting the dermal Shwartzman reaction, if it is due to interference with the clotting mechanism, is not due to any property which it has in common with dicumarol. Since they both inhibit the formation of thrombin it would appear that if heparin is effective because of its anticoagulant activity, the site of action in inhibiting the dermal Shwartzman is by preventing the conversion of fibrinogen to fibrin. Another possibility is that the inhibition by heparin of the dermal Shwartzman is an additive one, effects being exerted on both the formation of thrombin and also on that of fibrin, the two effects together being necessary for the inhibition of the dermal Shwartzman reaction.

The results in the present work are similar to those in another study(6) in which dicumarol and heparin were compared with regard to their effect on clot formation in isolated venous segments. It was found that heparin inhibited clotting while dicumarol did not. In spite of dicumarol's ineffectiveness, bleeding complications were much more severe in the dicumarol than the heparin-treated animals.

Summary and conclusions. 1. The effect of dicumarol on the inhibition of the dermal Shwartzman reaction was studied. 2. It was found that unlike heparin, dicumarol did not inhibit the dermal Shwartzman reaction.

1. Thomas, L. and Stetson, C. A., Jr., *J. Exp. Med.*, 1949, v89, 461.

2. Good, R. A. and Thomas, L., *ibid.*, 1953, v97, 871.

3. Stetson, C. A., Jr., *ibid.*, 1951, v93, 489.

4. Anfinsen, C. B., Boyle, E., and Brown, R. K., *Science*, 1952, v115, 583.

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Calcification XIV. Investigation of the Role of Chondroitin Sulfate in the Calcifying Mechanism.*† (21268)

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Mucopolysaccharides which contain sulfate are present in dentin and enamel(1), in bone (2), and in abnormal calcification of the arteries(3). Rubin and Howard(4) used meta-

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* These studies were presented in part before: (a) XIX International Physiological Congress, Montreal, Sept. 2, 1953; (b) New York Section, American Chemical Society, Feb. 12, 1954; (c) Am. Soc. Biol. Chem., Atlantic City, April 15, 1954 (*Fed. Proc.*, v13, 300 (1954).

† These studies were supported by: (a) contract (NONR 98701) between Office of Naval Research and the Jewish Hospital of Brooklyn; (b) grant from the National Institute for Dental Research of the National Institutes of Health, U. S. Public Health Service.

chromatic staining to show the presence of mucopolysaccharides in all the types of pathological calcification they studied, including calcinosis universalis, calcified bursitis, and renal stones of the calcium phosphate-carbonate type. They found no indication of polysaccharides in calcium oxalate and ammonium urate stones.

Under certain conditions the mucopolysaccharide chondroitin sulfate will react with collagen, the characteristic protein of bone and dentin, so vigorously as to replace absorbed dyes from the collagen(5). It has been suggested(6) that chondroitin sulfate may be essential for the calcification of hypertrophic rachitic cartilage.

Studies of the reversible inactivation of *in vitro* calcification have led Sobel and his colleagues to suggest that chondroitin sulfate may be the target of inactivating ions such as Be^{++}, Mn^{++}, Cu^{++}, Mg^{++}, Ba^{++}, Na^{+}, and Sr^{++}, and that Ca^{++} ions may reactivate the calcifying matrix by displacing these ions (7-10). It was to help clarify the role of the mucopolysaccharide (usually considered to be chondroitin sulfate) present in preosseous cartilage that the following work was undertaken.

Experimental. Materials and methods. Toluidine Blue "O"‡ was employed in a stock solution containing 10 mg/100 ml (1.58×10^{-4} M), and this was diluted as required, usually 1:4. Protamine sulfate§ was used as the 0.4% solution. A $CaCl_2$ stock solution prepared from the carbonate contained 500 meq/L of calcium and was diluted as required. It is important that a 150 meq/L solution of the $CaCl_2$ be tested by shaking with a rachitic bone section, to determine the presence of inhibiting ions which would interfere with reactivation. After this step, *in vitro* calcification of the rachitic section should show more intensive mineralization than the untreated control sections(10,11). Chondroitin sulfate was prepared from rachitic bone cartilage or from beef nasal septum cartilage.‖ The stock basal salt solution used to make up calcifying solution was kept for 2 or 3 weeks and contained 0.7 M NaCl, 0.05 M KCl, and 0.22 M $NaHCO_3$ per liter. It was kept in a pyrex bottle and diluted 10 fold when used in the calcifying solution. Redistilled ion exchange water was used. Other reagents used were: 95% phenol (95 g recrystallized phenol plus 5 g of distilled water), 38% formalin, absolute methanol, and 95% ethanol.

The bones used were the excised tibiae of rachitic Wistar strain albino rats. The animals were maintained on a rachitogenic diet for 3 or 4 weeks and then sacrificed by chloroforming. The diet consisted of 76% degerminated yellow corn meal, 20% wheat gluten, 3% $CaCO_3$, and 1% NaCl. Ion exchange water was accessible to the rats at all times. The excised tibiae were freed from adhering soft tissue, and the upper ends were sliced longitudinally into sections less than one mm in thickness. To insure homogeneous exposure to the calcifying and toluidine blue solutions, the sliced sections were suspended from glass hooks (made from melting point tubes) inserted in paraffined cork stoppers(11).

Rat tail tendon collagen was prepared from the tail tendon of Wistar rats. A one g sample of the tendon was freed of blood by repeated washing with distilled water, minced with a scalpel, and added to 100 ml of 0.04% acetic acid in distilled water. The resulting mixture was placed in the refrigerator overnight. Insoluble material was centrifuged off, and the slightly opalescent, viscous supernatant liquid thus obtained was employed in the experiments. Another collagen was prepared from the Achilles tendon of steer.¶ The skin was removed from the top and the tendon was cut into small pieces which were washed overnight with cold running tap water, covered with half saturated calcium hydroxide solution, and stirred mechanically for one hour at room temperature. Stirring was repeated 5 times with fresh calcium hydroxide. The protein left after extraction was rinsed with running tap water for 4 hours and then with distilled water. The final air dried residue consisted mainly of collagen with small amounts of elastin. The collagen-chondroitin sulfate complexes were prepared according to the method of Einbinder and Schubert(5) and a modification of Morrione's method(12). The control collagen was devoid of chondroitin sulfate as indicated by the lack of metachromasia.

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‡ Toluidine Blue "O" was kindly supplied by Hartmann-Ledden Co., C. I. No. 925, Philadelphia, Pa.

§ Protamine sulfate was obtained through the courtesy of E. R. Squibb and Sons, Control:28024, New Brunswick, N. J.

‖ For chondroitin sulfate from beef nasal septum cartilage we owe our thanks to Dr. S. Roseman of the University of Chicago, Chicago, Ill., and to Dr. E. Jorpes of the Karolinska Institute, Stockholm, Sweden.

¶ Collagen from the Achilles tendon of steer was kindly prepared by Dr. S. Natelson of the Rockford Memorial Hospital, Rockford, Ill.

The degree of calcification *in vitro* was evaluated by the method described by Sobel(13), Sobel and Hanok(9), and Goldenberg and Sobel(10). Lack of calcification was graded 0(0); maximum calcification 4(++++); the length of the line or deposit was designated by the +'s and its thickness or width by the number preceding the parenthesis. Each figure given in the tables was the result of 3 to 6 experiments; the fractional values result from the method of averaging. The degree of metachromatic staining (metachromasia) in the provisional zone of the epiphyseal cartilage was evaluated with a Leitz wide field stereo binocular microscope. Magnifications of 32X and 96X were usually employed.

Prior to treatment, bone sections from each animal were placed in glass spot plates and covered with 3% silver nitrate solution. They were then exposed to light from a tungsten filament lamp for 5 minutes. If healing was present the animal was discarded. Similar glass plates were used to examine the degree of metachromasia. The bone sections were covered with distilled water and examined by reflected light. Some sections were so intensely metachromatic that they appeared bronze instead of purple. However, if these were sliced with a razor blade and examined by transmitted light they showed a deep purple-red stain. After being stained with the dye, some sections were soaked in 95% alcohol prior to microscopic observation. This served to remove false metachromasia, as recommended by Montagna *et al.*(14). After one hour of soaking in toluidine blue solution, the metachromatic staining usually appeared as a pericellular aggregation about the cartilage cells. These observations confirm those made by Follis(15). The pericellular aggregation was not so evident in the sections treated with formalin, phenol, and methanol.

TABLE I. Influence of Toluidine Blue or Protamine to Calcium Ratios on Subsequent Calcification *In Vitro*.

Inactivation			Reactivation with 150 meq/L $CaCl_2$
Toluidine blue in sol., μM/L	$CaCl_2$ in sol., meq/L	Degree of calc., mean	Degree of calc., mean
39	.0	0 (0)	1.2(2+)
39	5.0	trace	
39	25.0	1 (+)	2.2(2+)
39	70.0	1 (2+)	
39	150.0	1.8(3+)	3 (3+)
39	100.0	1 (3+)	
1	139.0	1.3(4+)	
.6	144.0	1.7(4+)	
.0	.0	0 (0)	
.0	150.0	2 (4+)	
Protamine SO_4 in sol., %	$CaCl_2$ in sol., meq/L	Degree of calc.,* mean	Degree of calc.,* mean
.4	.0	0 (0)	1.3(2+)
.4	5.0		2.5(2+)
.4	10.0	1.5 (+)	
.4	25.0		2.3(3+)
.4	150.0	1 (4+)	3 (3+)

* Calcification, which was mostly below the surface, became evident when the sections were silver stained and then treated with alcohol and xylene.

Note: Following preliminary shaking in toluidine blue "0"—$CaCl_2$ solutions for one hr, and protamine—$CaCl_2$ solutions for 2 hr, sections were placed in calcifying solution for 18 hr at 37°C, pH 7.3 ± 0.07. Ca = 10 mg %; P = 5 mg %.

Inhibition and reactivation of calcifying ability. (A) Rachitic bone sections were shaken either in solutions containing different ratios of toluidine blue and $CaCl_2$ for one hour, or in solutions containing protamine and $CaCl_2$ for 2 hours. They were then placed for 18 hours at 37°C, in a calcifying solution with pH 7.3 ± 0.07; Ca = 10 mg %; P = 5 mg %. In some cases, after shaking with toluidine blue or protamine, the bone slices were reactivated with 150 meq/L of $CaCl_2$ and then placed in calcifying solution. The results are shown in Table I.

(B) Bone sections were incubated for 18 hours at 37°C in calcifying solution which had

TABLE II. Calcification *In Vitro* in the Presence of Toluidine Blue.

Toluidine blue, μM/L	Degree of calc., mean	Degree of metachromasia,* mean
39.0	0 (0)	+±
15.8	0 (0)	+
5.3	1 (4+)	doubtful
2.2	1.2(4+)	0
1.7	1.3(4+)	0
.0	1.5(4+)	0

* The degree of metachromasia was graded as follows: 0 = No purple (metachromatic) stain. ± = Broken thin line of metachromatic staining extending across the epiphyseal hypertrophic cartilage. + = Continuous thin line of metachromatic staining extending across the epiphyseal hypertrophic cartilage. 2+ = Continuous zone of metachromatic staining extending across the hypertrophic cartilage and about half way up. 3+ = Continuous zone of metachromatic staining extending across the hypertrophic cartilage and about three quarters of the way up. 4+ = Complete metachromatic staining of the hypertrophic cartilage.

the same composition as given in A, above, plus various proportions of toluidine blue. After incubation both the calcification and the degree of metachromasia were evaluated. Results are shown in Table II.

(C) Bone sections were treated with various reagents, then examined for metachromasia by staining with toluidine blue, and for calcifiability by incubating in calcifying solution. These were compared with sections which after treatment had undergone an additional shaking for one hour with 150 meq/L $CaCl_2$, before being examined for metachromasia and calcifiability. The results are given in Table III.

(D) When bone sections were treated with toluidine blue in the presence of calcium ions there was increased metachromasia up to 15 meq/L and then a gradual decrease. The results are shown in Fig. 1, and were confirmed with minor variations 6 times. Collagen prepared from Achilles tendon was condensed with chondroitin sulfate following the process of Einbinder and Schubert(5) and treated with toluidine blue in the presence of calcium ions, as in the case of rachitic bone sections. These results are also shown in Fig. 1.

The collagen-chondroitin sulfate complex, when shaken with 0.25 M $CaCl_2$ solution and then with 0.32 M phosphate solution, gave a silver stain similar to that found in *in vitro* calcification. The untreated complex did not exhibit this phenomenon, nor did collagen alone. When the complex was incubated in solutions employed for *in vitro* calcification there was a marked increase in the ash content. These changes did not take place when the complex was shaken first with phosphate solution and then with calcium solution.

When the rat tail tendon collagen-chondroitin sulfate complex, prepared by a modification of Morrione's method(12), was incu-

TABLE III. Metachromasia and Calcification by Various Treatments.

	Before shaking with $CaCl_2$		After 1 hr shaking with 150 meq/L $CaCl_2$	
Preliminary treatment	Metachromasia*	Calc.†	Metachromasia	Calc.
Basal salt sol., 2 hr, 37°C	3+	1(4+)		
" " " , 6 hr, "	2+	0 (0)		
" " " + 5 meq/L Ca^{++}, 2 hr, 37°C	4+	2(4+)		
" " " + " " " , 6 hr, "	4+	2(4+)		
Distilled water, 1 hr, room temp.	±	0 (0)	3+	2.5(4+)§
" " , 30 min., 97°C	2+	0 (0)	3+	2 (4+)§
Xylene, 1 hr	±	0 (0)	++±	2.5(4+)§
Ethyl alcohol 95%, 1 hr	2+	1 (+)		2 (3+)
" " " + solid $CaCO_3$, 1 hr		2(3+)		4 (4+)
Formalin, 38%, 1 hr	4+‡	0 (0)	4+‡	0 (0)
Methyl alcohol, absolute, 1 hr	3+‡	0 (0)	3+‡	0 (0)
Phenol 95% (95 g phenol + 5 g water), 1 hr	+	0 (0)	3+‡	0 (0)

* Metachromasia observed after preliminary treatment.

† Calcification observed after preliminary treatment when sections were placed in calcifying sol.

‡ Metachromasia was extremely intense and appeared as a velvety, bronze colored structure. Microtomed sections, viewed by transmitted light, appeared magenta colored.

§ See Sobel, A. E., and Hanok, A., Table VII (Ref. 9).

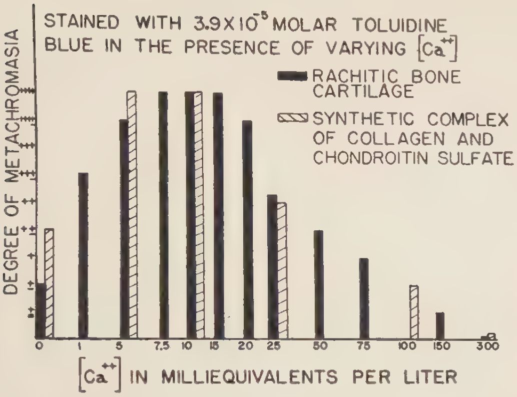

FIG. 1. Influence of calcium ions on metachromasia in *calcifying* rachitic cartilage and synthetic collagen-chondroitin sulfate complex.

bated in calcifying solution for 18-20 hours, washed, dried *in vacuo,* replaced in fresh calcifying solution for another 18-20 hours, and this process repeated 3 times, the ash content of the dried material was about 30%. The ash content of the collagen-chondroitin sulfate controls was less than 1%. X-ray diffraction patterns showed an apatite lattice. The X-ray studies were carried out by Dr. Albert Hirschman of the State University of New York College of Medicine at New York City (details of these studies will be published later).

(E) Rachitic bone sections were treated with 38% formaldehyde, with absolute methyl alcohol, or with 95% phenol, and examined for calcifiability and metachromasia. Calcifiability was destroyed but metachromatic activity survived as shown in Table III. In Fig. 2, where degree of metachromasia is plotted against the concentration of calcium ions in the staining solution, the metachromasia was maximal at 0.0 to 1.0 meq/L, and began to decrease when 20 milliequivalents of calcium ions or more were present in the solution. This is in contrast to calcifying cartilage (Fig. 1).

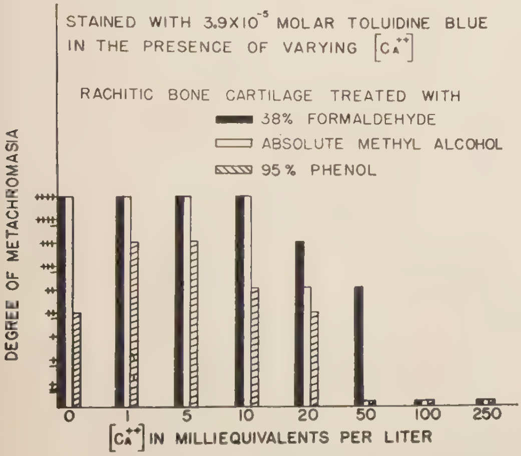

FIG. 2. Influence of calcium ions on metachromasia in cartilage *with destroyed calcifying mechanism.*

Discussion. The results given in Table I show that reversible inactivation is possible with both protamine and toluidine blue. Further, as is the case with inorganic ions, inactivation is a function of the inactivator to calcium ratio. The failure of Miller, Waldman and McLean(6) to observe that calcium ions prevented inhibition by toluidine blue was due to the fact that they used a lower calcium ion concentration (1 mM/L). From Table II it can be seen that inactivation is a function of the concentration of toluidine blue present in the calcifying medium. The higher the dye concentration, the greater the degree of metachromasia, and the less the calcification. Contrary to the finding of Sylven(2) concerning *in vivo* calcification, metachromasia can be found subsequent to *in vitro* calcification in the area that has been calcified *in vitro*. Both sets of data indicate that the organic cation competes with the calcium ion to combine with something presumably chondroitin sulfate, in the ossifying cartilage. Where the calcium ion is present in sufficient amounts there is calcification. Where it is replaced by toluidine blue there is less calcification or none at all.

Table III presents the results obtained when the dye and calcium ions were not competitive, but were used in different solutions to test for the effects of different reagents. When such varied reagents as distilled water, basal salt solution, xylene, and ethyl alcohol were used, the degree of calcification paralleled the degree of metachromasia. In the experiments with formalin, methyl alcohol, and phenol, however, as can be seen from Table III, calcifiability was destroyed and could not be reactivated with $CaCl_2$, whereas metachromasia was not affected or was enhanced. The latter reagents are protein denaturants, and it is therefore possible that the destruction of the calcifying mechanism results from an alteration of the protein in the chondroitin sulfate-protein complex. It may be tentatively inferred that the complex needed for metachromatic staining is less specific than that needed for calcification.

From Fig. 1 it can be seen that up to approximately 10 or 15 meq/L, calcium ion enhances the degree of metachromasia of rachitic bone sections. As the calcium concentration is raised further the degree of metachromasia slowly diminishes. Similar results were obtained with a synthetic complex of collagen and chondroitin sulfate. By contrast, the metachromasia exhibited by chondroitin sulfate alone was prevented or destroyed by calcium ion concentrations as low as 0.2 meq/L. This evidence, taken together with the fact that chondroitin sulfate-collagen complexes show calcification by the silver stain test, their increase in ash content and apatite x-ray diffraction patterns makes it seem highly probable that it is not the chondroitin sulfate alone, but a chondroitin sulfate-protein complex which is part of the calcifying mechanism. The behavior of these complexes with regard to metachromasia also parallels the behavior of rachitic bone cartilage. Collagen alone showed only the faintest blue stain with the dye, and no metachromasia.

The role of calcium ion appears to be at least 2-fold. As shown in Tables I and III, a loss in calcium ion (as in treatment with distilled water) results in both decreased calcifiability and decreased metachromasia. An increase in calcium, on the other hand, at first intensifies both metachromasia and calcifiability. Up to a certain concentration the calcium may cause a rearrangement to a more active chondroitin sulfate complex, possibly due to polymerization, as suggested by Sobel and Hanok(9). A further increase in the calcium ion concentration represents increased competition with the rearranged chondroitin sulfate complex. Hence, metachromasia now decreases, although calcifiability continues to increase. ** The metachromatic behavior of the mucopolysaccharide in rachitic bone sections (Fig. 1) is in contrast to that observed when a mucopolysaccharide, presumably chondroitin sulfate, prepared from rachitic bone cartilage or nasal septum cartilage, was treated in a similar manner. Here, calcium ions in concentrations as low as 0.2 meq/L prevented or destroyed metachromasia.

The mechanism of mineralization of the chondroitin sulfate-collagen complex appears to be first the formation of a calcium complex which in turn reacts with phosphate to form the apatite salt and this process is repeated. This mechanism is postulated from the findings that one obtains mineralization by shaking first with calcium then with phosphate but not in the reverse order.

Further experiments are planned with chondroitin sulfate from various sources combined with different proteins, among them keratin, the key protein in dental enamel, in an attempt to throw further light on the mechanism of calcification of teeth as well as bone.

Summary. 1. Toluidine blue and protamine, like inorganic cations, produce a reversible inactivation of the mechanism of calcification. This inactivation is a function of the inhibitor to calcium ratio. 2. Up to a concentration of about 15 meq/L, calcium ion in the dye solution increases the intensity of metachromatic staining. Above this concentration there is a gradual decrease of metachromasia. 3. Under certain conditions, the calcifiability of bone sections parallels metachromasia. But it is possible, by raising the calcium ion concentration, to destroy metachromasia while increasing calcifiability, or by treatment with certain protein denaturants, to destroy calcifiability while enhancing metachromasia. 4. Synthetic chondroitin sulfate-collagen complexes have been found to exhibit behavior similar to that of rachitic bone cartilage with regard to metachromasia and calcifiability. 5. Results obtained with chondroitin sulfate, collagen, and chondroitin sulfate-collagen complexes, indicate that such complexes behave in a manner homologous to the actual calcification mechanism, and support the hypothesis that they are involved in calcification.

We are indebted to Dr. Joseph Samachson for his

** Although in the presence of high calcium ion concentrations, metachromasia is not evident, the metachromatically active substance is still present in the rachitic cartilage, as shown by intense metachromasia if such bone sections are transferred to solutions containing toluidine blue in the absence of calcium ions.

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Calcification XV. *In vitro* Calcification of Rachitic Bone Cartilage of Thyroparathyroidectomized Rats.*† (21269)

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Several authors have shown that the secretion of the parathyroid glands has a direct action on bone metabolism(1-3). Although Jones(4) could find no significant difference in serum calcium or phosphate levels, or bone ash, of control and thyroparathyroidectomized animals, Pappenheimer(5) found rickets more difficult to produce in thyroparathyroidectomized rats. In addition, it has been shown that bone citrogenase activity is decreased in parathyroidectomized young rats(6).

The purpose of this investigation was to compare the *in vitro* calcification of rachitic bone cartilage of thyroparathyroidectomized animals with that of control rachitic animals, and indirectly to indicate whether the ability to calcify *in vitro* is related to bone citrogenase.

Methods. Albino rats of an original Wistar strain were kept on Bills Stock Diet(7). Each litter was divided into 3 groups: control animals on the stock diet; control animals on a rachitogenic diet(8); and thyroparathyroidectomized animals on a rachitogenic diet. (It was impossible to maintain thyroparathyroidectomized animals on the stock diet as a further control, since these animals died of tetany.) The diet employed had a calcium to phosphorus ratio of 10.3, the calcium being 1.27% of the diet. The diet had a complete vitamin supplement, exclusive of vit. D. Thyroparathyroidectomy was performed 24 hours after the experimental diet had been started, when the rats were 21 to 23 days old. The effectiveness of thyroidectomy was checked by the histologic examination of the pituitary gland as described by Selye(9). If

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TABLE I. Serum Calcium and Phosphate Determinations and Weight Change in Stock Control, Rachitic Control, and Rachitic Thyroparathyroidectomized Rats.

Group	No. of animals	Calcium, mg %*	Phosphate, mg %*	Wt change, g*
Stock control	4	8.8	8.9	96.1
Rachitic "	16	9.8	2.4	15.8
Rachitic thyroparathyroidectomy	25	10.5	2.7	8.5
Statistical analysis, P values†				
Comparing stock control with rachitic control		.20	<.01	<.01
Comparing stock control with rachitic thyroparathyroidectomy		.09	<.01	<.01
Comparing rachitic control with rachitic thyroparathyroidectomy		.29	.30	.07

* Mean values.

† P = Probability that difference between 2 means is due to chance. When $P \leqq .05$, difference between means may be considered statistically significant.

the so-called "thyroidectomy" cells were found it was assumed that parathyroidectomy was complete, since in the rat the parathyroids are embedded in the thyroid gland and are constant in number, one in each lobe(10). Animals failing to show the histologic changes in the pituitary were eliminated from the experimental data. After 25 to 28 days on the experimental regime, the animals were X-rayed to check for rickets, and then sacrificed by exsanguination. Serum calcium was determined by the micromethod of Sobel and Sobel(11), and serum phosphate by a modification of the Fiske and Subbarow method (12), allowing the use of 0.1 ml of serum. The statistical analysis of the serum calcium and phosphate determinations and the weight changes were made using Fisher's test of the significance of small samples(13). The bones were examined histologically for rickets. *In vitro* calcification studies were performed on slices of tibia suspended in a calcifying solution as described by Goldenberg and Sobel (14). The composition of the calcifying solution was as follows: NaCl, 0.07 M; KCl, 0.005 M; $NaHCO_3$, 0.022 M; $CaCl_2$, 0.0025 M; and $NaH_2PO_4 + Na_2HPO_4$, 0.00161 M per liter. The evaluation of the degree of calcification was that described by Sobel and his coworkers(15-17) (Table II). Lack of calcification was graded 0(0); maximum calcification 4(++++); the length of the line or deposit was designated by the +'s and its thickness or width by the number preceding the parenthesis. Each figure given in the tables was the result of 3 to 6 experiments; the fractional values result from the method of averaging. Magnifications of 32× and 96× were usually employed.

Results. It is immediately apparent from Table I that both the rachitic control and the rachitic thyroparathyroidectomized animals showed markedly depressed serum phosphate levels which, compared with the stock control animals, was statistically significant. This applies also for the ability to gain weight. However, no statistically significant difference was noted for serum phosphate or weight gain between the 2 groups of rachitic animals, nor for serum calcium among any of the groups of animals.

Examination of the bones histologically revealed normal bone growth and epiphyseal cartilage in the stock control animals and active rickets in both groups of animals on the rachitogenic diet.

In vitro calcification occurred in all animals on the rachitogenic diet, these animals having shown X-ray and blood chemical evidence of rickets. No *in vitro* calcification occurred in the epiphyseal cartilage of the stock control animals. While the estimates of the degree of *in vitro* calcification may vary slightly with

TABLE II.
In Vitro Calcification of Rachitic Bones.*

Group	Degree of calcification
Stock control	No rickets
Rachitic "	1.9 (3.6+)
Rachitic thyroparathyroidectomized	2.2 (3.6+)

* Mean values.

each observer, all estimates were made by the same observer, so that any error would apply to all groups. No significant difference was noted between the rachitic control and rachitic thyroparathyroidectomized animals.

Discussion. The ability of rachitic bone cartilage of thyroparathyroidectomized rats to calcify *in vitro* was comparable to that of the control rachitic animals, since both showed the same type and degree of response. Barnicot (1), Ingalls(2), and Munson(3) have presented evidence of a direct action of the secretions of the parathyroid gland on bone metabolism, and Perkins and Dixon(6) found a diminution of the bone citrogenase of young parathyroidectomized rats. The implication is that these factors do not cause changes in the intimate system responsible for calcification from inorganic solutions. The experiments in the present study utilized an inorganic substrate. It is possible that if the system were tested with organic substrates differences might be found. Thus, one may conclude that parathyroid hormones are not essential for the production of the "local factor" of calcification, and that their absence, in spite of the diminished bone citrogenase, does not block this mechanism.

The high calcium-low phosphorus diet employed in this experiment produces rickets by failing to supply sufficient phosphate for the deposition of bone salts. Since function of the parathyroid glands with such a large phosphate deficit is low(18), the concept that the primary action of the parathyroid gland is through the control of the excretion of phosphate in the urine(19), could not be tested. Our results are in accord with those of Jones (4), in that thyroparathyroidectomy had no protective action in conservation of phosphate, as reflected in the serum phosphate levels, or in lessening the severity of the rickets. Extirpation of the parathyroid glands has a profound effect on the serum calcium and phosphate when animals are maintained on a diet with a normal calcium to phosphate ratio. Moreover, it has been shown that parathormone increases the serum citric acid level(20), the response to comparable doses of parathormone being greater in thyroparathyroidectomized animals. Thus, an influence on serum calcium and phosphate levels and the ability to develop rickets may be elicited with a diet containing a lower calcium to phosphate ratio.

Summary. 1. Rickets was produced in rats thyroparathyroidectomized at 21 to 23 days of age as readily as in control animals. Both groups were on a rachitogenic diet with a calcium to phosphorus ratio of 10.3. X-ray routine histologic examination, and blood chemical analysis for calcium and phosphate were comparable in both groups of rachitic animals. 2. Rachitic bone cartilage from thyroparathyroidectomized rats calcified to the same degree as the rachitic bone cartilage from the controls. 3. Under the conditions of this experiment, neither the secretions from the parathyroid glands nor any enzyme system such as that involving bone citrogenase is essential for satisfactory *in vitro* calcification.

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Response of Serum Inorganic Phosphate to Insulin in Normal and Diabetic Subjects. (21270)

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Wigglesworth *et al.*(1) showed that injection of insulin produced a fall in blood inorganic phosphate in rabbits. A number of investigators have confirmed these findings, both in whole blood and in serum(2-11). Similar results were obtained by Harrop and Benedict (2) and by Perlzweig *et al.*(4) after oral administration of glucose to normal subjects. Bolliger and Hartman(8) pointed out that this phenomenon did not take place in pancreatectomized dogs and attempted to apply this finding to diagnosis of diabetes mellitus(12). We worked out a procedure which we termed "Delta G/Delta P test" for the study of carbohydrate metabolism based upon changes in blood sugar and serum inorganic phosphate* from their initial level(13). The test is carried out by injecting intravenously, at a speed of about 10 ml/minute, 1 ml/kilo body weight of a 50% glucose solution, taking samples for blood sugar and phosphate analysis before and 30, 45 and 60 minutes after injection. Subjects are kept at rest for one half hour before and during test. A Delta G at 45 minutes more than + 50 mg/100 ml is taken to indicate decreased glucose removal. In normal subjects Delta P at 45 minutes is greater than —0.30 mg/100 ml. In 45 normal subjects the *averages* were + 16 for Delta G 45′ and —0.67 for Delta P 45′(14). We used this test for classifying 54 diabetic patients into 2 groups: Group I, with a defective glucose-phosphate fall and group II, with a normal fall (15). We assumed that patients from the first group did not produce insulin (69% of patients tested), whereas patients from the second group (31%) were supposed to have a normal or near normal insulin reserve. Until now, only pregnancy(16) and fever and surgical stress(17) have been shown to inhibit at least partially the glucose-phosphate fall in non-diabetic subjects. We do not imply that insulin is the only factor concerned in the glucose phosphate fall. Thus, a glucose phosphate fall has been found to occur in pancreatectomized dogs(18) and in one pancreatectomized human being(19); in both cases, however, the blood sugar had to be raised above 500 mg/100 ml before the glucose phosphate fall took place.

Thus a working hypothesis consistent with available data would seem to be that glucose phosphate fall is mainly dependent upon insulin secretion by the pancreas, although factors other than insulin probably enter also into play in the production of the phenomenon. If this hypothesis were correct, one would expect a group of diabetics showing glucose phosphate fall and a group not showing it, to exhibit roughly equal insulin glucose responses. This would support the theory further, although it would not constitute definite proof for it. We investigated therefore the effect of insulin upon phosphate of diabetic patients previously classified according to their glucose phosphate fall, comparing

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* By "phosphate," we mean "serum inorganic phosphate," by "insulin-phosphate fall" the fall in serum inorganic phosphate following injection of insulin and by "glucose-phosphate fall" the fall in serum inorganic phosphate following injection of glucose.

TABLE I. Fall in Blood Sugar after 0.1 U/K Insulin. Means and standard errors, mg/100 ml.

	No. of subjects	Time after inj. of insulin				
		0′	15′	30′	45′	60′
Normal subjects	42	77 ± 2	–39 ± 2	–43 ± 2	–32 ± 1	–18 ± 3
Diabetics	21	180 ± 10	–24 ± 4	–39 ± 4	–53 ± 6	–53 ± 4
Diabetics, Group I	12	196 ± 13	–23 ± 7	–36 ± 5	–53 ± 9	–54 ± 5
" " II	8	154 ± 10	–26 ± 6	–44 ± 4	–54 ± 6	–51 ± 8

Normal vs. diabetics: Differences statistically significant at 0′, 15′, 45′ and 60′.
Group I vs. Group II: " " " " 0′ only.

results with those obtained in a normal control group.

Material and methods. Tests are carried out by injecting 0.1 unit of insulin/kilo body weight, intravenously. The subjects, 42 normal young adults and 20 diabetic patients were kept at rest for one half hour before and during test. They were in fasting conditions. Diabetic subjects had been given the Delta G/Delta P test; 8 had a normal glucose-phosphate fall (Group II) and 12 had a defective fall (taking as a limiting value —0.30 mg/100 ml) (Group I). Blood samples were taken from the antecubital vein before and 15, 30, 45 and 60 minutes after injection of insulin, and blood sugar and serum inorganic phosphate determinations were performed in duplicate(20-21). Hemolysis of samples was carefully avoided. The diabetics were all untreated before the test with insulin. Photometric readings were made on a Coleman Model 6 spectrophotometer. The statistical significance of the differences was established with the "t" test(22), the limit of significance being P=0.05.

Results. Results are shown in Tables I and II. Changes in blood sugar were less pronounced at 15 minutes in the diabetic than in the normals, but became more pronounced at 45 and 60 minutes. There was no difference in this respect between the 2 groups of diabetics. Changes in phosphate were more pronounced in normals than in diabetics, a fact which has not been mentioned previously, but there was no significant difference in the response of the 2 diabetic groups.

Discussion. It appears from the results that there are in diabetes mellitus certain factors which are capable of inhibiting the response of phosphate to insulin. Comparing the 45 minute value in the normal and in the diabetic group, shows that insulin phosphate fall in the latter is approximately 50% less than in the former. It is of interest that the diabetic patients classified according to glucose-phosphate fall do not show a difference when insulin is employed, suggesting that the subjects under study are homogeneous with respect to the insulin-phosphate response. This finding is consistent with the idea that the differences found after administration of glucose depend upon the capacity of the pancreas to produce insulin. A fairly numerous group of patients with diabetes show a normal glucose-phosphate fall, but in our experience, all patients who show this phenomenon had an initial blood sugar of less than 250 mg/ml (15), even though the defective phosphate fall may occur with initial levels less than 250 mg/100 ml; in Table I we may note that the average fasting blood sugar for Group I is only 196 mg/100 ml in the present series.

TABLE II. Fall in Serum Inorganic Phosphate after 0.1 U/K Insulin. Means and standard errors, mg/100 ml.

	No. of subjects	Time after inj. of insulin				
		0′	15′	30′	45′	60′
Normal subjects	42	3.55 ± .07	–.53 ± .07	–.95 ± .07	–.99 ± .07	–.75 ± .06
Diabetics	20	3.52 ± .23	–.29 ± .08	–.39 ± .12	–.51 ± .11	–.33 ± .07
Diabetics, Group I	12	3.53 ± .23	–.31 ± .13	–.37 ± .18	–.51 ± .14	–.25 ± .08
" " II	8	3.49 ± .47	–.27 ± .09	–.41 ± .09	–.51 ± .20	–.43 ± .14

Normal vs. diabetics: Statistically significant differences at 15′, 30′, 45′, 60′.
Group I vs. Group II: " " " " none.

A normal glucose-phosphate fall in persons with changes in glucose tolerance due to hepatic disease has been interpreted by Forsham and Thorn(23) as an indication of "peripheral utilization" of glucose. The only experimental support for this concept comes from the work of Pollack and his collaborators (24). These workers showed that glucose phosphate fall took place in the perfused hind limb of a dog; on the other hand, in a visceral preparation in which all the striated muscles had been removed, the phenomenon did not occur. Although the authors do not state it clearly, the conclusion appears to be based on a single experiment, in which the preparation was kept in good condition. Furthermore, there are grave objections to this type of experiment from the biochemical point of view. It has been shown by others that shock states tended to produce a rise in serum phosphate (25) which could well mask the glucose phosphate fall. Indeed, in Pollack's experiment, the serum phosphate level starts around 5.50 mg/100 ml. Finally, the concept of "peripheral utilization" is in opposition with experimental data indicating that the glucose phosphate fall mirrors an insulin discharge and its effect upon most tissues, including the liver (25-26). From the data available, it appears that the only case in which the glucose-phosphate fall has taken place in the absence of the pancreas has been in the work of Levine *et al.* with blood sugar levels above 500 mg/100 ml during several hours(18), and in one pancreatectomized patient(19). As above mentioned in clinical cases of diabetes we never found a normal glucose-phosphate fall with fasting blood sugar over 250 mg/100 ml.

We believe therefore that, as a working hypothesis, it may be admitted that diabetic patients who show a normal glucose-phosphate fall produce insulin, possibly even in quantities greater than are secreted under normal conditions. However, the possibility that other modifying factors might be present during glucose administration which were not present when insulin was given without glucose is not eliminated by the present data.

Summary. Intravenous insulin tests (0.1 U/kg of body weight) were carried out in 42 normal subjects and in 20 diabetics, blood sugar and serum inorganic phosphate being determined before and 15, 30, 45 and 60 minutes after administration of insulin. The blood sugar fall was found to be less in the diabetic patients at 15 minutes, similar in both groups at 30 minutes and more pronounced in the diabetic at 45 and 60 minutes. The phosphate fall was more pronounced in normals than in diabetics. At 45 minutes for instance, the average fall in diabetics was about 50% that found in normals. If diabetics are classified in 2 groups according to their normal or defective phosphate response to glucose no difference is seen between the 2 groups with regard to their response to insulin. It is suggested that the phosphate fall which takes place in a number of diabetics after the injection of glucose may be in part dependent upon an insulin secretion by the pancreas. It is pointed out that the experimental data thus far accumulated do not permit the assumption that the phosphate fall after glucose represents "peripheral utilization" of the carbohydrate.

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Formazan Formation Compared to Phosphatase Activity in Human Serum.* (21271)

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Malignant tissue is stained more intensely with tetrazolium salts than normal tissue according to Straus and his coworkers(1) and Black *et al.*(2). This difference apparently is caused by the higher concentration of the glycolytic enzymes in abnormal tissue(3-7). The question arises whether such enzymatic changes in the tissue are reflected in the blood or serum of the patient. The results of our work indicate no correlation between the reducing power of serum enzymes and malignancy. Instead tetrazolium reduction in serum is apparently correlated with phosphatase activity.

Materials and method. Triphenyltetrazolium reagent: One g of 2,3,5-triphenyl-2-H-tetrazolium chloride (Eastman), 6.7 g potassium dihydrogen phosphate, and 350 ml of 0.1 *N* sodium hydroxide are diluted to one liter with distilled water. The pH of this reagent is 7.2. Two ml of the triphenyltetrazolium reagent, 6.0 ml distilled water, and 0.2 ml of serum are mixed in a test tube, and incubated at 38° C for 24 hours in the dark, because exposure to ultraviolet light itself causes reduction. The tubes are then cooled in an ice-bath and 8.0 ml of acetone is added. This mixture is stoppered and shaken well to dissolve the red formazan. If proteins precipitate and cause cloudiness, the mixture can be centrifuged. A sample of aqueous acetone mixture is pipetted off and placed in a cuvette. The color intensity is measured in a Beckman Model B Spectrophotometer at 485 mμ. Acetone, which has been shaken with an equal volume of water, is used as the reference solution or blank. The alkaline and acid phosphatase analyses were run according to a spectrophotometric modification of the Bodansky(8) and Gutman(9) methods.

Results. Using the method of Straus(1), our results showed that various malignant tumor tissues do not always show a deep red staining, and some normal tissues, such as mucosa of the gastro-intestinal and urinary tracts, hepatic parenchyma, renal cortex, uterine endometrium, and the parenchyma of the testis show a deep staining by formazan.

Sera from patients with cirrhosis, Cushing's syndrome, renal rickets, hypo- and hyperparathyroidism showed as great a reduction in transmission as those patients with neoplasms. However, the lowest per cent transmissions were obtained from patients with cancer of the

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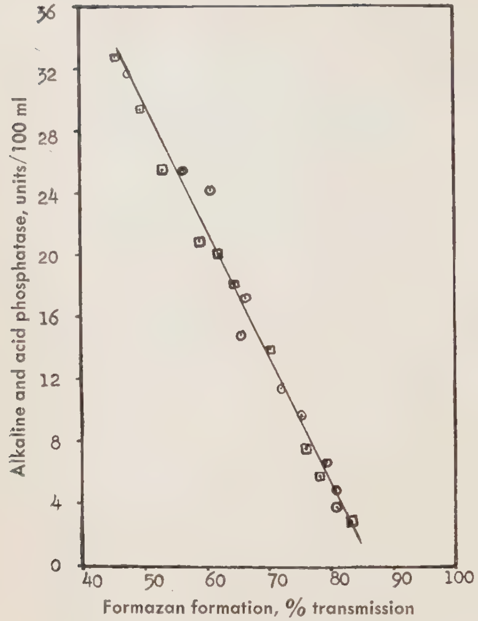

FIG. 1. Phosphatase activity vs. formazan formation. % transmission at 485 $m\mu$. Alkaline phosphatase in Bodansky Units *(dotted square)*. Acid phosphatase in King-Armstrong Units *(dotted circle)*.

prostate and osteogenic sarcoma, neoplasms with high serum phosphatase activity.

This led us to investigate phosphatase activity. Fig. 1 shows the correlation between phosphatase activity of serum and the formation of formazan from triphenyltetrazolium.

Summary. The reduction of tetrazolium by serum is not a specific test for malignancy. It seems however to be correlated with the serum alkaline and acid phosphatase activity.

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Factors Controlling Color Change in the Tree Frog, *Hyla versicolor* Wied.

(21272)

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Control of color change in amphibians has been studied by many workers; material published prior to 1943 was summarized by Parker(1). According to Parker's summary, frogs respond to cool dark environments by release of melanophorotropic hormone and darkening of integument, whereas light and warmth result in melanophore contraction and light colors through retention or nonsecretion of hormones by the pituitary. Many chemical factors also have melanophorotropic actions and produce darkening of integument, either by stimulating release of melanophorotropic hormone by action on pituitary, or by direct action upon melanophores(2). Recently the study of factors which control color changes in various species has become of considerable practical importance. Johnsson and Högberg(3), Sulman(4) and Thing(5) reported melanophorotropic effects of ACTH

TABLE I. Progressive Color Changes in *Hyla versicolor* and Numerical Assignments.

Color	No.
Green	1
Olive	2
Mottled	3
Beige	4
Brown	5

in amphibians. These studies, based upon hypophysectomized *Rana*(3,5) and light-adapted Amphibia(6,7,8), or untreated Hylid frogs(9) provide simple techniques for the assay of adrenocorticotropic substances.

In searching for suitable Nearctic frogs for this assay, we have studied environmental factors controlling color change of *Hyla versicolor* and *Hyla cinerea*(9).

Materials and methods. Frogs were maintained in groups of 5-8 in 600 ml beakers, with about 100 ml of water. Light was provided by a 100 watt frosted bulb placed exactly one foot from near side of beaker, and for darkness, beakers were covered with a double layer of cloth toweling. Experiments were carried on in a darkened cold-room (3-5°C) or at normal laboratory temperatures (20-24°C). Color changes are regular in occurrence, passing from a light green to a dark brown; they have been arbitrarily divided into 5 stages. For quantitative analyses each stage has been assigned a numerical value (Table I). Responses have been summarized by using simple mean values termed the "color index" (CI). This correlates closely with the melanophore index of Hogben and Slome(10).

Results. Effects of constant light: In preliminary experiments frogs were placed into constant light for 2 weeks. All frogs were then beige or brown (CI = 4.7). After 72 hours under normal diel conditions they were again subjected to continuous light for one week; the color index remained 4.7. Controls for the following experiments were maintained in constant light; color indices remained around 4.7 throughout.

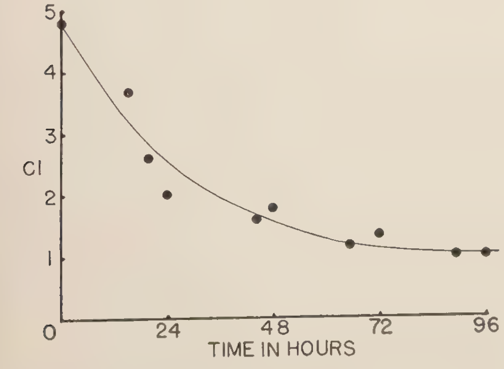

FIG. 1. Decrease of color index (CI) with increasing period of dark-adaptation. The curve was fitted visually.

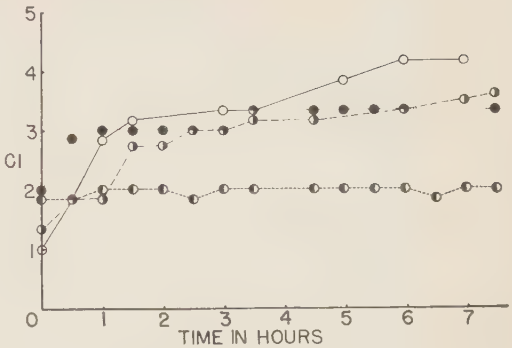

FIG. 2. Light effects after varying periods of dark-adaptation. Solid circles and dotted lines: 24 hr; half circles open to right and short dashed line: 48 hr; half circles open to left and long dashed line: 72 hr; hollow circles and solid line: 96 hr; ¾ circles; common point of 2 or more lines.

Effects of constant darkness: Constant darkness for varying periods of time resulted in lowered color indices. To determine the relationship between time and color index, groups of 6 frogs were darkened for varying periods. The decrease of color index with extended periods of darkness is shown in Fig. 1. Although about 48 hours of darkness was enough to cause a complete response (green color) in most frogs, over 72 hours were necessary to turn all frogs green.

Light-adaptation after varying periods of darkness: At the completion of above experiments the frogs were returned to constant light. No consistent relation between dark-adaptation time and change toward light-adapted condition was found. CI values after 24, 48, 72 and 96 hours of dark-adaptation have been plotted in Fig. 2, and are typical of responses obtained. The 72 and 96 hour groups showed more complete, though less rapid, returns toward control condition, than the 48 hour frogs which showed essentially no reaction.

Effects of temperature: The above data suggested that light tended to darken frogs and darkness to lighten them. As cold causes darkening in most frogs, 8 light-adapted frogs were refrigerated at 3-5° C, and 5 were darkened at regular laboratory temperatures (Fig. 3). Cold had no obvious effect upon already expanded melanophores, while controls at nor-

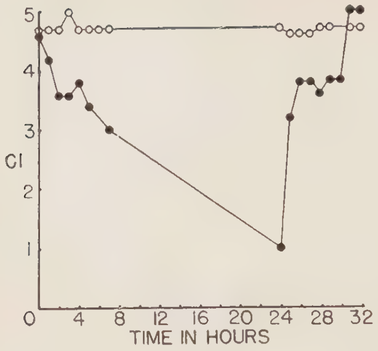

FIG. 3. Effects of darkness and temperature on light-adapted frogs. Group 1 (hollow circles) placed in darkened cold room; Group 2 (solid circles) darkened at room temperature. Both groups returned to light at room temperature at 24 hr.

mal laboratory temperatures showed decrease of CI. Return to constant light and normal laboratory temperatures had no effect upon cold-dark-adapted frogs, whereas warm-dark-adapted frogs showed return toward control conditions. Two groups of 6 frogs each dark-adapted for 72 hours, were then placed in refrigerator for 24 hours. Five frogs died during experiment; color index values for the remainder have been plotted in Fig. 4, together with controls, maintained in the dark at normal laboratory temperatures. Thus low temperatures also cause integumentary darkening.

Discussion. These data indicate that *Hyla versicolor* responds to darkened laboratory environments by contraction of integumentary melanophores and to light or low temperatures by the expansion of these cells. Expansion of the melanophores in frogs is controlled by pituitary hormone(1) known variously as intermedin, melanophore expanding hormone, chromatophore hormone, etc. It has been suggested that this hormone is a member of an adrenocorticotropic hormone complex(11), although separation of the factors is possible under certain conditions(12).

Data presented here suggest that release (or secretion) of melanophorotropic hormone is largely under the control of light, which acts through the eyes of the frogs to stimulate the pituitary (see number 1 on effects of blinding). In darkness, hormone is retained and melanophores contract. Low temperatures also darken the integument, presumably by stimulating hormone release.

Hyla versicolor is highly unusual, responding to light in a manner opposite to other species. Kohler(13) has suggested that color changes in lower vertebrates may be explicable on the basis of Selye's concept of stress(14). The stressor stimulates the release of pituitary ACTH which then results in melanophore expansion. If this explanation is valid, light must act as a stressor in *Hyla versicolor* but not in a congeneric form, *Hyla arborea,* which turns green in the light(6). Dampness, a necessity for amphibian life, would also be a stressor(1). Though Selye's GAS and color-changes may be related phenomena, the relationship is certainly not as simple as Kohler implies.

The unexpected control of melanophorotropin release in *Hyla versicolor* may be the result of selection for darkly colored frogs during the day when the frogs are inactive and take refuge in recesses in bark and other such niches. In such places a green frog would be highly conspicuous and perhaps subject to predation. Thus selection may have favored the darker daytime color, and hormone release in response to light.

Summary. Hyla versicolor responds to light by melanophore expansion, and to darkness by contraction. The completeness of contraction depends upon duration of the period of dark-adaptation, generally more than 72 hours are required for all frogs to turn green. The change to brown after dark-adaptation is vari-

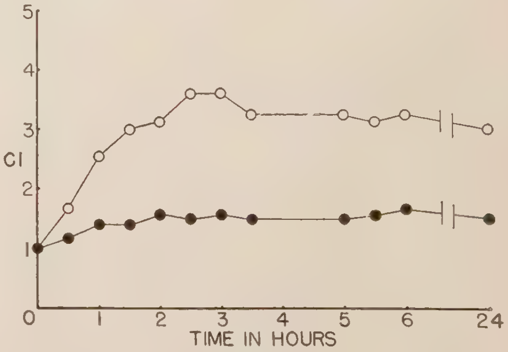

FIG. 4. Effects of cold after 72 hr dark adaptation at room temperature. Solid circles: frogs maintained in darkness; hollow circles: frogs transferred to cold-room.

able and no consistent relationship has been found between length of darkness and length of recovery period. Temperatures of 3-5° C darken frogs as does light. Light and cold appear to be stimuli which cause release of melanophorotropic hormone (ACTH?) from the pituitary gland. Light-induced changes, opposite to those shown by other frogs, seem explicable on the basis of natural selection during the diurnal period when these frogs are inactive.

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Effect of Immunity on Resistance to Infection in Irradiated Mice and Rats.* (21273)

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Bacteria which are common inhabitants of the intestinal tract are frequently found in blood and various organs of lethally irradiated mice(1-3). The efficacy of antibiotics in reducing mortality(4,5) and the effect of certain of the organisms on survival time(2,6) show that these infections cause or contribute toward death in the irradiated animal. Irradiation reduces the defenses against infection, in part, by decreasing the number of circulating granulocytes and by interrupting the production of antibodies. Granulocyte count has recently been shown to be quantitatively related to survival after midlethal radiation(7). In the present experiments the effect of immunity acquired prior to irradiation on ability to withstand bacterial challenge and on survival following lethal radiation are examined.

Procedures. Male N.I.H. littermate mice and Sprague-Dawley rats from the N.I.H. animal colony, maintained on a diet of Purina Laboratory Chow, were used in these studies. The animals were caged individually from

TABLE I. Effect of Immunization on Survival of Animals Experimentally Infected with *Proteus* after LD_5 Irradiation.

Exp.	Immunizing antigen	No. of animals	% surviving	P†	Days from chal. to death, ± S.E.
Mice: 6 immunizing inj., challenge 5 days after irradiation					
1	0	47	17		3.3 ± .5
	Prot. Kf 7	50	66	<.001	10.3 ±1.2
2	0	40	2		1.1 ± .1
	Prot. AM	39	31	<.01	5.2 ± .6
3*	0	39	0		1.2 ± .3
	Prot. AM	40	63	<.001	6.5 ± .7
Rats: 6 to 7 immunizing inj., challenge 4 days after irradiation					
A	0	43	42		2.2 ± .4
	Prot. Kf 7	60	90	<.001	9.8 ±3.4

* Challenge organism was *Proteus* Kf 7 in this experiment.

† Significance of differences in survival of Exp. and Control groups by the method of χ^2.

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* Supported in part by the Department of Defense.

TABLE II. Effect of Immunization Schedule on Survival of Mice Challenged with *Proteus* Kf 7 (1:1) after Exposure to 475 r.

	Immunizing injections					
		Wk pre-irradiation				
Exp.	No.	1st inj.	Last inj.	Conc.*	No. mice	% surviving 4 days†
4	0				44	5
	1	3		2:1	45	64
	6	3	1	2:1	45	71
5	0				40	8
	1	4		1:1	40	45
	6	4	2	1:1	39	67
6	0				30	3
	1	7		1:2	30	63
	6	7	1	1:2	30	80

* Heat killed suspensions of 24 hr cultures in concentration indicated.

† Survival of nonirradiated, nonimmunized challenged mice: *Exp. 4,* 73%; *Exp. 5,* 50%; *Exp. 6,* 33%.

the beginning of immunization and were 15 to 20 weeks old at the time of irradiation. Irradiation was carried out with a 200 Kvp X-ray unit operating at 20 ma with 0.25 mm Cu and 0.51 mm Al added filtration. Mice were irradiated 10 at a time in a partitioned plastic container, the target distance being 50 cm and dose rate in air 55 r per minute. The rats were irradiated in groups of 12 in a partitioned pressed wood box, with target distance 70 cm and dose rate 23 r per minute. The irradiation groups, and in mice the litters, were appropriately distributed among the various treatment groups. Procedures used for bacterial culture have been described elsewhere(8). Briefly, subcultures of the lyophilized organisms were cultured for 24 hours at 37°C in horse meat infusion broth and centrifuged, and the organisms suspended in physiological saline. Vaccines were prepared by heating the suspensions at 60°C for one hour and were given subcutaneously 6 times over a period of 3 weeks, with one week elapsing between the final injection and irradiation (with the exceptions noted in the Tables). The challenge consisted of a single subcutaneous injection of a suspension of living organisms for mice and an intraperitoneal injection for rats. Animals alive 28 days after irradiation were counted as survivors except in Table II where survivors were counted 4 days after challenge. In the challenge experiments irradiation alone (475 r for mice, 500 r for rats) killed only about 5% of the animals.

Results. When mice were treated with a series of immunizing injections prior to LD_5 irradiation, resistance to a challenging infection with the same organism or mixture of organisms was increased as shown in Tables I-III. The differences between vaccinated and nonvaccinated groups were statistically significant in all experiments with *Proteus* alone and with mixed bacterial species. The rats of *Exp. A,* Table I (which represents pooled results of 3 smaller experiments) also responded favorably to the immunizing process. Im-

TABLE III. Effect of Vaccination on Survival of Mice Exposed to 475 r (LD_5) and Experimentally Infected with Single or Mixed Bacterial Species Isolated from Lethally Irradiated Littermates.

				% surviving		
Exp.	No. per group	Organism*	Immunizing inj.†	Irr. control	Exp.	P
7	24–30	A (*Proteus*)	5	4	40	$<.01$
		B (*E. coli*)	5	33	60	$<.1$
		C (*Paracol.*)	5	33	44	$<.7$
		ABC	5	0	62	$<.001$
8‡	39–40	D (*Pseudo.*)	6	60	78	$<.1$
		ABCD	6	20	67	$<.001$
9§	39–40	ABDE (α-strep.)	6	5	38	$<.001$

* Organisms used for challenge and their incidence in lethally irradiated littermates: A, *Proteus mirabilis* (20–31%); B, *E. coli* (15–38%); C, *Paracolon coliforme* (0–14%); D, *Pseudomonas aeruginosa* (3–4%); E, α-streptococcus (14–46%).

† Heat killed 24 hr cultures of each organism mixed in equal parts and injected in concentrations ranging from 1:8 to 1:1 over a period of 2 wk with 1 wk elapsing before irradiation.

‡ Of 20 nonirradiated challenged controls in each group all survived.

§ " 30 " " " 22 survived.

TABLE IV. Survival and Occurrence in Post Mortem Heart Blood of Bacterial Species Used for Vaccination in Lethally Irradiated Mice (625 r).

Antigen*	No. of mice	% surviving	% positive†
Single species			
Control	45	4	15
Vaccinated	110	3	10
Mixed species			
Control	136	34	41
Vaccinated	139	28	34

* *Proteus, E. coli, Paracolon,* as single species (Exp. 7); mixed species as in Exp. 7, 8 and 9 of Table III.

† Post mortem heart blood positive for bacterial species used in immunizing injections.

munization with *Proteus mirabilis* of mouse origin (AM) was effective against challenge with *Proteus vulgaris* of human origin (Kf 7, from the Kauffman collection) as shown in *Exp. 3,* Table I. In addition to increasing the number of challenged animals that survived 28 days, the immunization consistently prolonged the survival time of those that died.

The results of one and 6 immunizing injections, given according to the schedules and concentrations indicated, are compared in Table II. The groups given 6 injections had a slightly higher survival than those given a single injection. No quantitative relationship was observed between serum agglutinin titer and the effect of immunization on resistance to bacterial challenge.

The beneficial effect of vaccination with the bacterial species commonly found in the blood of lethally irradiated mice (Table III) suggested that such vaccination might also increase survival following lethal irradiation. Groups parallel to those of the experiments shown in Table III (with the exception of D, *Pseudo., Exp. 8*) were given the same course of immunization but were exposed to 625 r and were not challenged. The pooled results from these groups are shown in Table IV. In no case was any degree of protection evident, nor was there a significant reduction in the number of mice showing blood cultures positive for the bacterial species used in the vaccines.

Since a beneficial effect of antibodies on survival in these experiments must be implemented by the action of phagocytic cells, granulocyte counts after lethal (600 r) and sublethal (475 r) irradiation were compared (Fig. 1). During the period of maximum depletion median counts were about 170 per cu mm in the sublethally irradiated mice and 30 per cu mm in the mice exposed to 600 r (57% mortality).

Discussion. It has long been known that specific antibodies persist following whole-body X-irradiation(9), and the present experiments show that antibodies formed prior to sublethal irradiation increase resistance to bacterial challenge. Hatch has recently reported similar results(10). The fact that the vaccinations neither increased survival nor brought about significant shifts in the specific infections of mice exposed to 625 r suggests either that the effective antibody content was lower after 625 r than after 475 r or that a content which affords protection in sublethally irradiated animals is totally inadequate in animals exposed to the higher radiation dose. At such doses the number of circulating granulo-

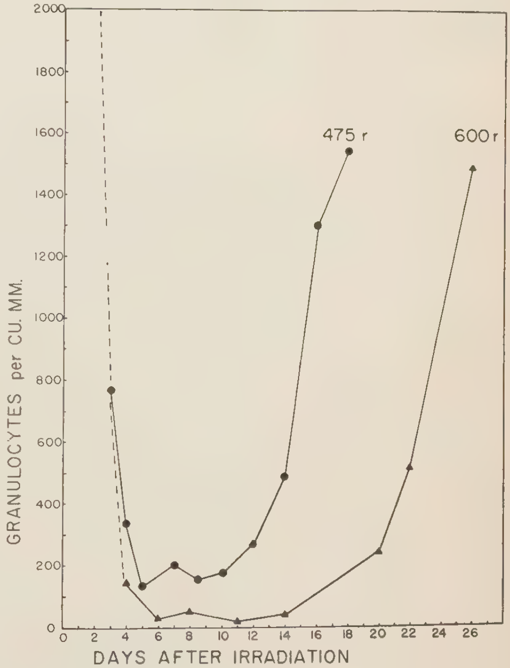

FIG. 1. Granulocyte counts of N.I.H. mice following 475 r or 600 r. The 600 r data are from a single experiment, each point representing the median of 8 to 12 counts. The 475 r data are compiled from 8 experiments (635 counts), each point representing the weighted average of the group medians.

cytes is severely depleted over a relatively long period of time and this with possible impairment of phagocytic function(11,12) may account for the difference in effectiveness of antibodies under the 2 conditions studied.

Summary. Immunization with *Proteus* prior to LD_5 irradiation increased resistance of mice and rats to challenge with the living organism. Immunization with mixtures of the bacteria which occur most frequently in post-mortem blood increased resistance to challenge in mice. Immunization with these mixtures did not increase survival in mice exposed to LD_{65-95} radiation.

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Effect of Xenoplastic Adrenal Transplants upon Limb Regeneration in Normal and Hypophysectomized Newts (*Triturus viridescens*). (21274)

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The following experiments arose from investigations concerned with the role of the pituitary as a requisite for regeneration in newts (1,2,3,4,5). When, in addition, it was recently shown that the administration of ACTH and also of several cortical extracts to hypophysectomized newts, otherwise incapable of regeneration, reinstated in these animals the capacity for regeneration(6), it became probable that the adrenal glands might be an important or even essential hormonal factor in limb regeneration. Unfortunately, the participation of the adrenals in regeneration is not demonstrable by adrenalectomy because of the diffuseness of these glands in urodeles. As one of the methods to explore the premise that purely adrenal products may replace the missing pituitary in limb regeneration, this research has investigated the effects of xenoplastically transplanted frog adrenals into previously hypophysectomized newts.

Materials and methods. All the experiments were performed upon normal adult or upon hypophysectomized newts (*Triturus viridescens*) and the adrenal transplants were obtained from adult *Rana pipiens* and *R. palustris* kidneys by dissection immediately before transplantation. For control purposes muscle transplants have been used. The frog adrenal or muscle transplants were introduced under a skin pocket of the heavily vascularized lower jaw. Using this technique several series of transplantations were made. In a first group the hypophysectomized newts received a single adrenal transplant; in another group one adrenal was implanted at the time of amputation and additional frog adrenals were implanted at the fifth and also at the tenth day following amputation. In a final group the transplantation, limited to one adrenal only, was delayed until the sixteenth day after amputation at which time the second limb of the newt was freshly amputated. All limbs were studied histologically and the heads of

TABLE I. Effect of Frog Adrenal Transplants upon Limb Regeneration in Hypophysectomized Newts.

No. of animals	1 adrenal simultaneous with amputation	1 adrenal with first amputation, 1 or 2 additional transplants 5 or 10 days later	1 adrenal 15 or 16 days after amputation
2	++		
4	0		
8		++	
3		0	
3		*	
20			++

++ Advanced, typical regeneration of the limb. * Arrested regeneration. 0 Typical non-regeneration.

the animals which regenerated were sectioned to verify the completeness of pituitary removal.

Results. Effects of adrenal transplants on the regeneration of normal animals. Single adrenal transplants to control series of normal animals had no observable effect either upon the usual pattern of regeneration or upon the general behavior of these animals. As might be expected the endocrine system with intact pituitary is far more competent than the hypophysectomized system to compensate for abnormal hormone balances.

Regeneration of limbs of hypophysectomized newts with adrenal transplants. As may be seen in Table I, only 2 hypophysectomized animals were observed to regenerate under the influence of single adrenal transplants and many cases of typical non-regeneration were recorded in this group. Somewhat better success was obtained with increased doses of adrenal tissue, particularly when additional adrenal transplants were performed 5 or 10 days later. When viewed in the context that hypophysectomized animals never regenerate, regeneration in more than one-half of the animals which received adrenal transplants on the tenth day following amputation, appears to demonstrate that purely adrenal products are competent to replace the pituitary in regeneration in histologically verified absence of this gland. A final series involving transplantation delayed after amputation, however, provides a more consistent picture. A single adrenal was transplanted into these hypophysectomized newts 15 or 16 days after the first amputation at which time a second limb was

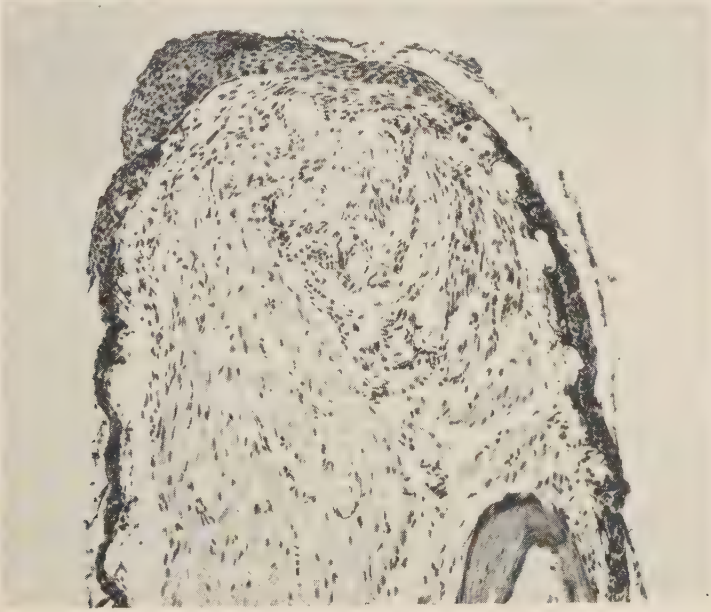

FIG. 1. Section through right forelimb of a hypophysectomized newt, the limb pictured having been amputated at the time of adrenal transplantation. Limb fixed 23 days after amputation exhibits typical non-regeneration indicated by the dermis with darkly staining chromatophores invading the area and separating the cut tissues from the epidermis.

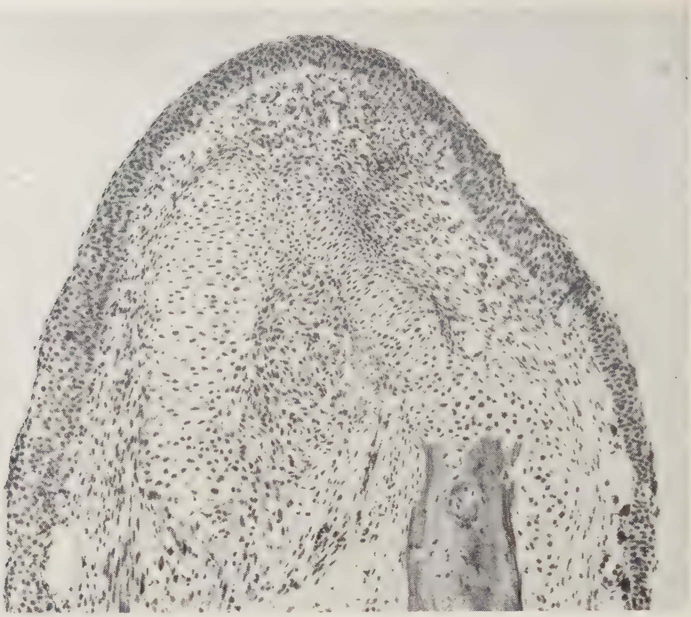

FIG. 2. Photomicrograph of a section through the regenerating left forelimb of the same animal as represented in Fig. 1, but which was amputated sixteen days previous to introduction of a single frog adrenal transplant. Limb was fixed 39 days after amputation and shows advanced regeneration with differentiation of skeletal formations.

amputated. In all 20 cases in which transplantation was performed on the fifteenth or sixteenth day after amputation, regeneration was initiated in the limb amputated previous to the transplantation, whereas typical nonregeneration was observed in the opposite limb of each animal, amputated at the time the adrenal transplant was introduced. The photomicrograph of Fig. 1 from a hypophysectomized newt represents a section through the right forelimb which was amputated simultaneously with the introduction of an adrenal transplant. Sixteen days earlier, the left limb (Fig. 2) of the same animal was amputated simultaneously with the extirpation of the pituitary and was fixed after 23 days of regeneration. These figures illustrate well the results of the experiment while recapitulating, as it were, the general procedure followed. Fig. 1 shows that a limb from a hypophysectomized newt may not regenerate when a frog adrenal is transplanted simultaneously with the amputation. However, the same adrenal transplant brings about typical regeneration in the other limb of this animal which has been allowed to "regenerate" previous to transplantation for 16 days in histologically proved absence of the pituitary, a result which has been confirmed in all the cases of this series.

Observations on hypophysectomized animals with muscle transplants. Frog muscle (generally *M. sartorius*) transplanted into the lower jaw under conditions identical with those described for adrenal transplants produced no regeneration whatsoever in these hypophysectomized newts. Clearly then, the introduction of pieces of foreign tissue other than adrenal, at whatever time, does not reinstate the capacity for regeneration in hypophysectomized newts, an observation which as this is written has been confirmed by identical results with other tissues.

Discussion. The above results provide an affirmative answer to the question whether purely adrenal products are capable of acting in the same way as exogenous ACTH, cortisone or experimental stress (unpublished results from this laboratory) to reinstate in hypophysectomized animals the ability to regenerate. This investigation, therefore, is interpreted as supporting the hypothesis that the stress of amputation in naturally regenerating animals is mediated by the anterior pituitary which elicits a hyperadrenal response, comparable to that which has been observed in mammalian endocrinology under conditions of stress(7). That the previous work with injected cortisone(6) produced regeneration because of the hormone supplied and not because of contaminants is shown by the success of the above experiments in which adrenal tissue may induce regeneration of limbs in hypophysectomized newts. That the effect of the operation of transplantation itself is negligible is demonstrated by the fact that single or plural muscle transplants and lately also other tissue introduced simultaneously or successively in respect to amputation never result in regeneration within limbs of hypophysectomized newts. While, then, frog adrenal transplants in hypophysectomized newts may perhaps not function normally over extensive periods of time even when administered in larger amounts, they will nonetheless induce regeneration if introduced during a period when the amputated limb of a hypophysectomized newt has become susceptible to adrenal therapy. The unexpected success of delayed single adrenal transplants in initiating good regeneration in amputated limbs of animals deprived for two weeks of their pituitary deserves special attention. This observation implies a relatively short period of effective function for the adrenal transplant and it also suggests that the balance between regeneration and non-regeneration is a labile one, a fact which tallies with other observations in the field of regeneration.

Summary. 1. Our previous experiments showed that recuperation of regeneration in limbs of hypophysectomized newts may be obtained by replacement therapy with ACTH and with adreno-cortical hormones. We tested the hypothesis that an hyperadrenal state is necessary for regeneration. The study of effect of xenoplastically transplanted single or multiple adrenal frog glands upon regeneration of the fore limb in previously hypophysectomized newts was the method used. Control experiments showing that frog muscle tissue and also other tissues transplanted under similar conditions had no effect upon the non-regeneration typical for hypophysectomized newts indicated that xenoplastic transplants *per se* produced no observable effects upon regeneration. Frog adrenals, however, while not affecting regeneration in normal animals were found capable of restoring to hypophysectomized newts the ability to regenerate amputated limbs otherwise lost after extirpation of the pituitary. 2. Hypophysectomized animals are more susceptible to adrenal therapy 10 to 20 days after amputation than during earlier periods, since it is when transplanted at this time that frog adrenals most consistently produced regeneration. These results while not clearly understood, point to the existence of a critical stage within the post-amputational phases of regeneration when regeneration is particularly susceptible to adrenal hormones, endogenous or experimentally supplied. 3. Heretofore unsuspected involvement of adrenal secretions in limb regeneration of newts is thus demonstrated and the pituitary-adrenal synergism discovered for conditions of "stress" in mammals acquires a new importance for regenerative processes in amphibia.

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Mechanism of Action of Mercurial Diuretics II.* (21275)

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In a previous paper(1) it was reported that at the height of diuresis produced by a therapeutic dose of mersalyl, kidney slices showed no change in oxygen consumption. The succinic oxidase activity of kidney homogenates as studied by the method of Schneider and Potter(2) was likewise unaffected. In this paper we are reporting on the effect of mercurial diuretics on an –SH containing coenzyme, coenzyme A, utilizing kidney slices and the method of Wieland and Rosenthal(3). All these methods involving the use of homogenates and slices are open to the criticism that the mercury might be diluted by the medium, that is, it might not remain bound to the cell constituents. We have therefore sought to study the effect of mercurials on the Krebs cycle *in vivo* by using the "sequential blocking" method introduced by Potter(4). This author utilized fluoroacetate-poisoned rats and studied the change in citrate accumulation using malonate as a second block, which acted on a different link in the Krebs cycle. If the mercurials were to inhibit one of the steps of the citric acid cycle one would expect a decrease in accumulation of citrate in the kidney of fluoroacetate-poisoned animals, as was observed with malonate. In the previous paper(1) we have also stated that even if a therapeutic dose of the mercurial were to inhibit an enzyme system, it does not necessarily follow that that particular enzyme system is responsible for the reabsorption of sodium or chloride in the renal tubules. More direct evidence would be to produce diuresis by a specific inhibitor of the enzyme system in question. We have thus studied the effect of sodium malonate, an inhibitor of succinic dehydrogenase, on intact hydrated rats and compared it with that of an equiosmotic dose of an "osmotic" diuretic like potassium nitrate, and an equimolar dose of diethylmalonate. The latter compound is interesting because it inhibits the succinic oxidase system not only in kidney slices but also in the intact kidney as shown by the method of "sequential blocking."

Finally, we are reporting in detail on the method of measuring mercurial diuresis in the rat which was briefly mentioned earlier(1), stressing in particular the narrow therapeutic range of mercurial diuretics in the rat. Recent statements(5,6) that inhibition of succinic oxidase is responsible for mercurial diuresis may be questioned on the following grounds: The amounts of mercury used to demonstrate enzymatic inhibition had not been tested to determine whether they were the minimum doses which produced maximum diuresis. Furthermore, no evidence was presented that the time at which the animals were killed corresponded to the period of maximum diuresis. It is known that succinic dehydrogenase is one of several enzyme systems containing sensitive —SH groups and that it can be inhibited by mercury compounds. If, therefore, sufficient mercury were added to such a system *in vitro,* or injected *in vivo* one would naturally expect an inhibition of the succinic oxidase activity.

Methods. Local male and female albino rats were used(7). For the mercurial assays 7-month-old male rats were used which were obtained from Tierzüchterei Brünger, Halle i.W., Germany. All rats intended for diuretic studies were kept on a constant diet for at least one week prior to the day of the experiment. Food but not water was withheld after 5 P.M. on the previous day. Three rats were put in one cage, and if possible, the 3 were selected so as to have the total weight in each cage the same. The assay was performed on several consecutive days, each day including the total dosage range of the diuretic. Salyrganic acid, 125 mg, was dissolved in 2.5 cc N/10 NaOH as a stock solution. The mercurial was injected *intravenously* into the tail vein

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in a volume of 0.2 cc per 100 g body wt, using a 27 gauge needle. The intravenous injection was followed immediately by the intraperitoneal administration of isotonic solution of sodium chloride, 5 cc/100 g body wt. The urine output was measured at hourly intervals for the next 12 hours and again at the end of 24 hours. Unless otherwise stated, the above procedure was also utilized in the metabolic experiments involving diuresis, in particular with regard to the method of hydration. Fluoroacetate, 3 mg/kg, was administered intraperitoneally 30 minutes after the mercurial in one set of experiments and after 3 hours in another set. In both cases the animals were killed 2 hours after treatment with fluoroacetate. The organs were analysed for citrate by Natelson's(8) modification of the pentabromacetone method as previously described(7). Dry weights of tissues were measured in experiments involving diuresis. In the set of experiments in which fluoroacetate was injected 3 hours after the mercurial we used the acetic anhydride-pyridine method of Saffran and Denstedt(9). Here, 2 blanks were run, one on a pure 5% trichloroacetic acid solution and another on a trichloroacetic acid tissue extract as intended for the determination of aconitic acid. This method compares favorably with the pentabromacetone method, but as the authors themselves state(9), it cannot be used in the presence of large amounts of organic acids. For experiments involving the use of added or injected metabolites we have used the pentabromacetone method. Although the amounts of citrate are expressed in terms of mg per gram wet tissue, an appropriate correction was made for the change in percentage dry weight of kidneys of animals treated with mersalyl. *Coenzyme A* activity was measured by using the procedure of Wieland and Rosenthal(3), who added oxalacetate and acetoacetate to kidney brei and isolated citrate. We used slices instead of brei, and $MgCl_2$ instead of $BaCl_2$. Slices of 100 mg were suspended in 2 cc solution and the mixture incubated with shaking at 38°C for an hour. It is important to use a small quantity of slices of uniform thickness since the oxalacetate cannot be added in unlimited amounts. Trichloracetic acid was then added to make a final concentration of 10% and the filtrate used for citrate determination. The medium was always prepared without oxalacetate, the latter being added just before the experiment. This is important as some oxalacetate changes spontaneously into citrate in a solution containing $MgCl_2$(10). In addition, blank experiments containing all constituents except tissue slices, were run simultaneously and the nonenzymatic citrate production subtracted from the corresponding values obtained with slices. Each rat served as its own control. The left kidney, removed under ether anesthesia, was analysed after saline injection, and the right kidney, removed a week later, was analyzed 2 hours after the injection of mersalyl; control experiments having shown that there was no difference in the activity of either kidney removed at such different intervals. *Malonate* experiments were performed on intact rats, and kidney and heart slices of the rat. When intact animals were used, sodium malonate, diethylmalonate, and malonic acid diamide were injected subcutaneously in a dose of 1.15 millimoles per 100 g body wt. Thirty minutes later, fluoroacetate (3 mg/kg) was injected intraperitoneally and the animal killed after 2 hours for the measurement of citrate in tissue. When the diuretic action of the malonate derivatives was studied, 5 cc isotonic solution of sodium chloride per 100 g body wt were injected simultaneously with the malonate. The effect of malonate on tissue slices was studied by the conventional Warburg method. The

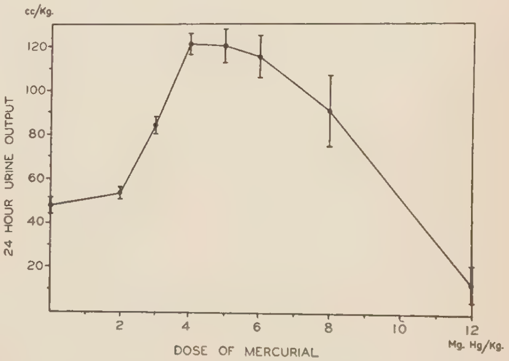

FIG. 1. Effect of increasing doses of mersalyl on 24-hr urine output of hydrated rats. Bars indicate standard error.

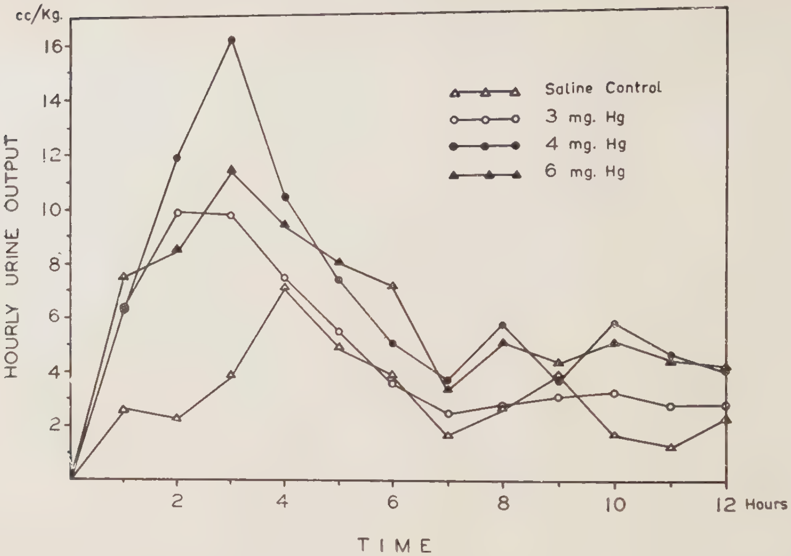

FIG. 2. Mersalyl diuresis in rats as a function of time.

medium used was Krebs-Ringer phosphate according to Webb *et al.*(11) with or without 0.02 M succinate. The inhibitor was added from the side-arm after the respiration had attained a steady rate (20 min.) in an atmosphere of oxygen. Respiration was measured for another 30 minutes.

Results. Effect of varying doses of Mersalyl on Diuresis and Tubular Histology. Fig. 1 illustrates the dose-response relationship for mersalyl. Each point on the curve is an average of 5 experiments and included 15 rats. The bars indicate the standard error. It will be seen, in agreement with many other unrecorded experiments, that with 4 mg of mercury per kilo, maximum diuresis is reached. It is unnecessary to use higher dosage. Amounts of 8 mg Hg/kg produce less diuresis, and 12 mg produces almost complete anuria. All rats receiving 12 mg of mercury and 50% of those receiving 8 mg mercury/kg died within a week after the injection. Kidneys removed 24 hours after the injection showed damaged tubules with all the doses of mercury used, the extent of the injury being proportional to the dose. With the highest dose used —12 mg Hg/kg—not only the proximal but also part of the distal convoluted tubules were involved. In the animals that survived, complete tubular regeneration took place within two weeks. Since in our experiments pure mersalyl was injected intravenously, it is quite possible that tubular damage may be more extensive than if the mercurial were injected subcutaneously and with theophylline. Furthermore, different strains of rats may react differently. Fig. 2 represents diuresis as a function of time. It is seen that maximum

TABLE I. Effect of Mersalyl (4 mg Hg/kg) on Accumulation of Citrate in Heart and Kidney of Fluoroacetate-Poisoned Rats.

No. of animals	Sex	Age, mo	Time between mersalyl and fluoroacetate inj. (hr)	Method	Citrate in heart (μg/g wet tissue), mean ± S.E.	Citrate in kidney (μg/g wet tissue), mean ± S.E.
9	♀	18	3	Acetic anhydride	1453 ± 68	865 ± 40
8	♀	18	S*	”	1402 ± 54	894 ± 70
9	♂	3.5	3	”	1227 ± 42	1024 ± 80
9	♂	3.5	S	”	1244 ± 47	977 ± 80
10	♂	4	½	Pentabromacetone	1310 ± 44	1250 ± 95
10	♂	4	S	”	1393 ± 29	1396 ± 78

* S = Saline controls.

TABLE II. Effect of Sodium Malonate, Ethyl Malonate and Potassium Nitrate on Diuresis in Rats. (All rats hydrated by injecting 50 cc saline/kg intraperitoneally.)

Substance	No. of rats	Mean urine flow in cc/kg body wt/hr					24 hr urine flow in cc/kg
		1st hr	2nd hr	3rd hr	4th hr	5th hr	
Saline controls	9	2.2	2.2	4.7	4.6	6.1	83
Sodium malonate, 1.15 mM/100 g	33	23	15.5	9.1	5.6	4.7	89
KNO_3, 1.72 mM/100 g	12	17.6	13.3	12.5	8.1	7.4	84
Ethyl malonate, 1.15 mM/100 g	12	2.7	5.9	3.2	4.7	4.1	69

diuresis is attained 2 to 3 hours after the administration of mersalyl.

Effect of mersalyl on citrate accumulation in fluoroacetate poisoned animals. Table I summarizes the results obtained by 2 different citrate methods and by the use of several batches of rats at different times. The amount of citrate in tissues varies, among other things, with the nutritional state and age of the animals, hence controls should be taken from the same batch. Since these tests were performed on starved animals, an increase in the amount of citrate in the heart is noted in agreement with the finding of Potter(4) and also a decrease in the amount in the kidney, as compared with values previously reported from this laboratory(7). The table shows that there was no significant decrease in the accumulated kidney citrate as would have happened had the mercurial, like malonate, blocked one of the steps of the citric acid cycle. The coenzyme activity of kidney slices as measured by Wieland's reaction: oxalacetate + acetoacetate → citrate, also does not appear to be diminished in rats at the height of mercurial diuresis. In 8 control rats treated with saline, 5 cc per 100 g body wt, the left kidney synthesized 8.4±0.38 mg citrate per gram wet tissue per hour, and the right kidney, removed a week later, synthesized 8.9±0.41 mg. In the next group of 8 rats, the left kidney synthesized 8.8±0.37 mg citrate per g per hour after intraperitoneal saline injection whereas the right kidney, removed a week later, synthesized 9.4±0.56 mg after the intraperitoneal injection of salt solution and intravenous mersalyl.

Malonate experiments. Sodium malonate is strongly diuretic when injected subcutaneously into hydrated rats in a dose that substantially inhibits the kidney succinic dehydrogenase, that is, 1.15 mM per 100 g body wt(4). This effect appears to be a purely osmotic one, since the injection of an equiosmotic dose of KNO_3, 1.72 mM, also produces strong diuresis. However, this comparison is at best semi-quantitative as diuresis depends on the amount of osmotic diuretic excreted rather than the amount injected. Convincing proof that the diuretic action of sodium malonate is osmotic and not enzymatic is furnished by the fact that an equimolar dose of diethylmalonate which inhibits the kidney succinic dehydrogenase to the same extent as sodium malonate (Table III) had no diuretic action. This is shown in Table II.

Table III illustrates the action of malonate in inhibiting the succinic oxidase activity of the kidney *in vivo,* confirming the findings of Potter *et al.*(4). Here it is seen that half the regular dose of sodium malonate is less effective than the full dose. Ethylmalonate has the same inhibitory action on the kidney as an equimolar dose of sodium malonate.

Ethylmalonate, but not malonic acid diamide, also inhibits succinate oxidation by renal tissue but has less effect on slices of heart.

TABLE III. Effect of Malonate Derivatives on Accumulation of Citrate in Heart and Kidney of Fluoroacetate-Poisoned Rats. 4 rats in each series.

Substance	Citrate in heart (μg/g wet tissue), mean ± S.E.	Citrate in kidney (μg/g wet tissue), mean ± S.E.
Na malonate 1.15 mM/100 g	1170 ± 71	443 ± 40
Na malonate 0.57 mM/100 g	998 ± 9	863 ± 32
Diethyl malonate 1.15 mM/100 g	897 ± 28	468 ± 63
Malonic diamide 1.15 mM/100 g	1097 ± 46	1970 ± 97

TABLE IV. Effect of Sodium Malonate and Ethyl Malonate on Oxygen Consumption of Rat Heart and Kidney Slices with and without Succinate (0.02 M).

Tissue	Substrate	No inhibitor	Sodium malonate, .02 M	Ethyl malonate, .02 M
Heart	Succinate	$Qo_2^{O_2} = 36$	$Qo_2^{O_2} = 6.1$	$Qo_2^{O_2} = 15.5$
Kidney	—	14.3	4.5	2.5
"	Succinate	58.5	15.8	6.2

This is shown in a typical experiment illustrated in Table IV. In both tissues the malonic ester appears to undergo some hydrolysis, for the pH at the end of the experiment was lower than at the beginning: 6.3 in the heart and 5.6 in the kidney, in the presence of succinate. Webb *et al.*(11) have shown that a pH of 6.3 does not affect oxidation in heart slices. However, the greater inhibition of succinate oxidation in kidney slices by ethylmalonate as compared to sodium malonate may well be due, in part at least, to the difference in pH.

Discussion. If Potter's concept of "sequential blocking"(4) be valid, and if the observed decrease of accumulated citrate in fluoroacetate-poisoned rats after malonate be due to the (*in vivo*) inhibitory action of malonate on the renal succinic oxidase system, then the diuresis produced in rats after therapeutic doses of mersalyl cannot be due to an inhibition of one of the Krebs cycle enzymes. Otherwise, mersalyl would have acted like malonate by decreasing the accumulation of citrate in fluoroacetate-poisoned rats.

Ethylmalonate is as effective as sodium malonate in decreasing the amount of accumulated citrate in fluoroacetate poisoned rats. However, ethylmalonate, unlike sodium malonate, does not produce diuresis in rats. This is evidence that sodium malonate is diuretic by virtue of its osmotic effect rather than its inhibition of activity of succinic oxidase.

The results reported in this and the previous paper(1) do not exclude the possibility that mercurial diuresis is due to interaction of mercury with –SH groups or –SH enzymes in the kidney. We feel, however, that such –SH groups ought to be sought elsewhere than among the Krebs cycle enzymes or coenzymes.

Summary. 1) A method for measuring mercurial diuresis in the rat is described. The effect of varying intravenous doses of mersalyl on hydrated rats has been studied. Maximum diuresis is obtained with a dose of 4 mg Hg/kg and the height of diuresis is reached in 2 to 3 hours. The extent of tubular damage is proportional to the dose of the mercurial. No rats survived a dose of 12 mg Hg/kg and only 50% survived a dose of 8 mg. In the animals that survived, complete tubular regeneration took place within two weeks. 2) The results reported in this paper do not support the view that mersalyl, in therapeutic doses, has any effect on the oxidative enzymes of the Krebs cycle. Mercurial diuresis may be due to interaction of mercury with –SH groups or –SH enzymes in the kidneys. It is felt, however, that such –SH groups be sought elsewhere than among the Krebs cycle enzymes and coenzymes.

We are indebted to the Sterling-Winthrop Research Institute for a generous supply of salyrganic acid.

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Comparison of Myotrophic and Androgenic Activities of 19-Nortestosterone Cyclopentylpropionate, Androstanolone, and Methylandrostenediol. (21276)

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From the Research Laboratories, Upjohn Co., Kalamazoo, Mich.

A recent publication from these laboratories (1) described the effect of 19-nortestosterone and some of its esters on causing increase in size of the rat levator ani muscle with relatively little stimulation of the seminal vesicles. Of the esters, 19-nortestosterone cyclopentylpropionate was considered most worthy of further study on the basis of potency and preferential myotrophic activity. The present paper describes the comparison of 19-nortestosterone cyclopentylpropionate (NTCP)[*] with 2 other steroids reported to have preferential anabolic activity, methylandrostenediol (MAD)[†] and androstanolone[‡](2,3), with regard to their abilities to stimulate seminal vesicles and levator ani in the castrate male rat.

Method. The test method was described in detail previously(1). Weanling male rats are castrated and the test compounds are injected subcutaneously in oil for 21 days. Seminal vesicle and levator ani muscle weights(4) are used as indices of androgenic and myotrophic activity. The dose-response data are plotted on semilogarithmic graph paper and potency ratios calculated by the method of Irwin(5). In calculating the data we choose straight-line portions of the dose-response curve of the unknown steroid which are most nearly parallel to the dose-response curve of the standard, testosterone propionate (TP).

Results. Methylandrostenediol. MAD was tested at a dosage range of from 50 to 1500

TABLE I. Comparison of Activities of Testosterone Propionate, 19-Nortestosterone Cyclopentylpropionate, Methylandrostenediol, and Androstanolone on the Seminal Vesicles and Levator Ani Muscles of Castrate, Weanling Male Rats.

						Organ wt (mg/100 g body wt)			
		No. animals		Body wt		Sem. ves.		Lev. ani	
Treatment	Daily dose (μg)	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Oil	—	14	15	142	153	5	6	23	24
Testosterone propionate	25	5	5	163	157	46	39	39	36
	50	10	10	160	166	94	85	57	55
	100	8	10	168	172	134	137	77	73
	250	10	10	171	169	193	179	80	81
	500	5	5	168	166	196	204	98	92
19-Nortestosterone cyclopentylpropionate	12.5	5	5	154	157	6	6	36	34
	25	5	5	156	158	5	6	52	47
	50	10	10	166	167	10	9	69	61
	100	5	5	163	171	25	36	90	88
	125	5	5	167	163	22	22	85	73
	250	5	5	171	174	67	66	86	89
	500	5	5	167	166	125	137	93	104
Methylandrostenediol	50	1		158		4		26	
	100	2		154		5		23	
	250	10		154		12		28	
	500	10		157		39		35	
	1000	5		201		78		47	
	1500	5		160		106		69	
Androstanolone	50		10		154		13		34
	100		10		155		33		39
	250		10		158		55		53
	500		10		158		106		78

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[*] Paper describing preparation and physical and chemical properties of this compound is in press (*J. Am. Chem. Soc.*): Babcock, J. C., R. A. Donia, B. A. Johnson, and A. C. Ott.

[†] 17α-methyl androst-5-ene-3β,17β-diol.

[‡] 17β-hydroxy androstan-3-one.

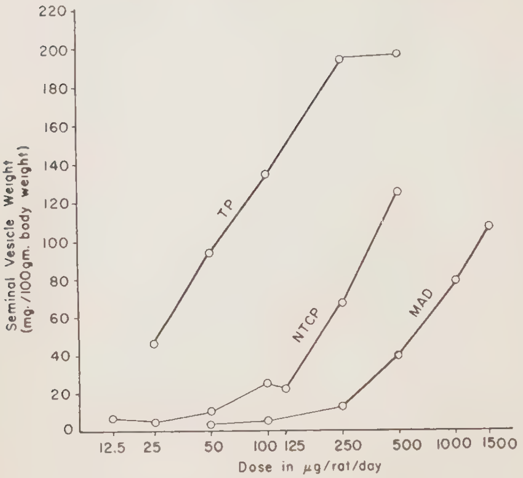

FIG. 1. Seminal vesicle weights of rats treated with testosterone propionate (TP), methylandrostenediol (MAD), and 19-nortestosterone 17β-cyclopentylpropionate (NTCP).
Portions of data used in statistical analysis presented in bold lines in all 4 figures.

μg/day. At doses less than 250 μg there was essentially no androgenic or myotrophic response (Table I, Fig. 1 and 2). At doses above 500 μg (5 mg/cc) the steroid crystallized from the oil and it was necessary to give it as a suspension. Statistical analysis of the results of these tests showed MAD to have a myotrophic potency[§] of 2.2% and an androgenic potency of 2.1% giving a myotrophic/androgenic ratio of 1.0. NTCP studied simultaneously had potencies of 160% and 15% giving a ratio of 10.7 (Table II). These data indicate that MAD has no preferential myotrophic activity, since both myotrophic and androgenic activities are of the same low order.

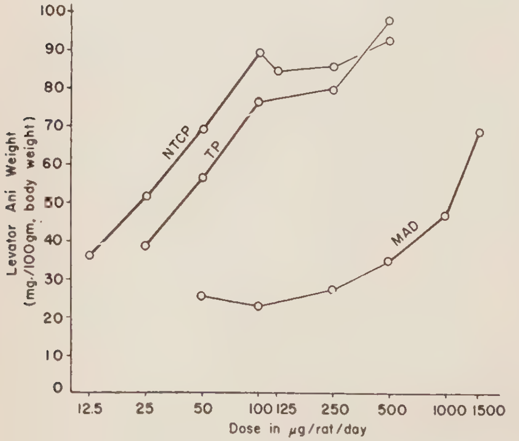

FIG. 2. Levator ani muscle weights of rats treated with testosterone propionate (TP), methylandrostenediol (MAD), and 19-nortestosterone 17β-cyclopentylpropionate (NTCP).

Androstanolone. This compound showed activity at a dosage range of from 50 to 500 μg/day (Table I, Fig. 3 and 4). Statistical analysis of the data from these tests showed androstanolone to have a myotrophic potency of 21.8% and an androgenic potency of 13.2% with a resultant myotrophic/androgenic ratio of 1.7. NTCP on parallel tests had a ratio of 8.5[‖]. Thus of the two steroids currently available for therapy where anabolic effect is desirable and "virilizing" effects are undesirable, androstanolone had higher potency than MAD, as well as a more favorable myotrophic/androgenic ratio. NTCP, on the other hand, while causing only slightly more seminal vesicle stimulation than androstanolone, produced about 6 times greater myotrophic response and thereby a much higher ratio of activities. NTCP had a mean ratio of 9.5 for the two comparison assays.

The use of the levator ani muscle as an indicator of general anabolic activity has been open to some question because of the slight possibility of its being stimulated only as a "secondary sex organ." That it does reflect the state of the whole organism with respect to protein build-up or break-down is strongly indicated by the recent work of Eisenberg and Gordan(6) who showed levator ani atrophy in states associated with protein catabolism. Stafford, Bowman, and Olson(7) showed that the nitrogen retention activity of the steroids tested in this study is in the same order as the levator ani-stimulating activity reported here. It is believed that these circumstances materially strengthen the case for the levator ani as an index of protein metabolism.

Although the nitrogen retention experiments (7) are not subject to the same quantitative analysis as the levator ani data, it is interest-

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[§] Results expressed as % of similar activity of standard (TP).

[‖] Five multidose assays comparing NTCP and TP have been performed. Anabolic/androgenic ratios ranged from 7.7 to 10.8 with a mean ratio of 9.3.

TABLE II. Myotrophic and Androgenic Potencies and Myotrophic/Androgenic Ratios of 19-Nortestosterone Cyclopentylpropionate, Methylandrostenediol, and Androstanolone.

Test substance	Androgenic* potency	Myotrophic* potency	Myotrophic/androgenic ratio
Testosterone propionate	100	100	1.0
Methylandrostenediol	2.1	2.2	1.0
19-Nortestosterone cyclopentylpropionate	15	160	10.7
Androstanolone	13.2	21.8	1.7
19-Nortestosterone cyclopentylpropionate	17.6	150	8.5

* Myotrophic and androgenic potencies expressed as percentage of activity of testosterone propionate.

ing to consider their results in light of the androgenic activity reported in this study. The doses used in the nitrogen retention series were NTCP .25 mg, androstanolone 2.0 mg, methylandrostenediol 2.0 mg. Considering the seminal vesicle stimulation reported here, the relative androgenicity of these doses is: NTCP .03, androstanolone .32, methylandrostenediol .04. Since NTCP caused much greater retention of nitrogen than either of the other compounds, while having the lowest androgenic potency, it is obvious that by this criterion, as well as by the levator ani end-point, NTCP is a more effective anabolic compound with low androgenic activity than either MAD or androstanolone.

Summary. 1. Three steroids with protein anabolic activity were tested in parallel with testosterone propionate to compare their relative properties of stimulating the levator ani muscles and seminal vesicles of weanling castrate male rats when injected subcutaneously in oil solution or suspension. The androgenic and myotrophic potencies of the test compounds, as well as their myotrophic/androgenic ratios, were calculated by analysis of significant portions of the data. 2. The androgenic potencies of these steroids, expressed as percentages of the potency of testosterone propionate, were: methylandrostenediol 2.1, androstanolone 13.2, 19-nortestosterone cyclopentylpropionate 16.2. 3. The myotrophic potencies, expressed as percentages of the potency of testosterone propionate, were: methylandrostenediol 2.2, androstanolone 21.8, 19-nortestosterone cyclopentylpropionate 155. 4. The myotrophic/androgenic ratios of these compounds are, therefore: methylandrostene-

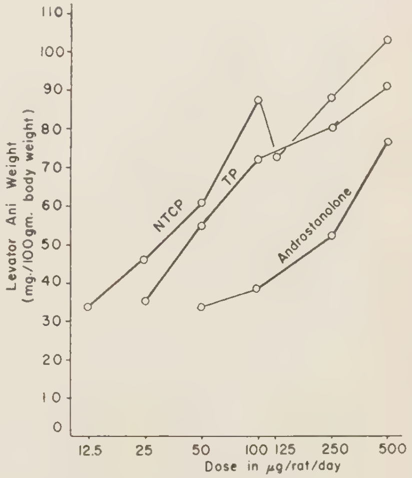

FIG. 4. Levator ani muscle weights of rats treated with testosterone propionate (TP), androstanolone, and 19-nortestosterone 17β-cyclopentylpropionate (NTCP).

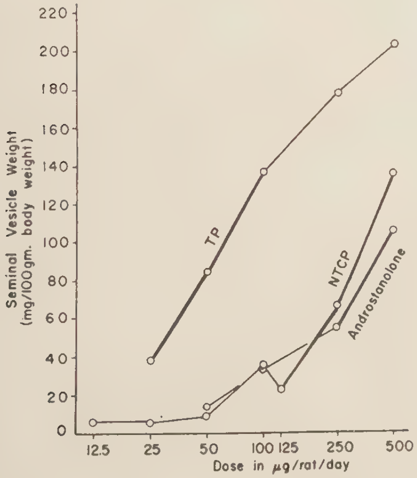

FIG. 3. Seminal vesicle weights of rats treated with testosterone propionate (TP), androstanolone, and 19-nortestosterone 17β-cyclopentylpropionate (NTCP).

diol 1.0, androstanolone 1.7, 19-nortestosterone cyclopentylpropionate 9.5.

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Vitamin B_{12} Serum Levels and Diabetic Retinopathy.* (21277)

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Methods for the determination of vit. B_{12} activity in serum have been reported(1,2,3), although the measured activity may not be due to this vitamin *per se*. Recently, a modified procedure was described(4) which permits the estimation of as little as 10 micromicrograms of activity of this crystalline vitamin. The availability of this method prompted us to compare the serum vit. B_{12} levels of diabetics with or without retinal lesions. These findings which will be reported in this communication, should be of particular interest since we have shown earlier(5) that following an intramuscular test dose of vit. B_{12}, diabetic subjects with retinopathy excrete significantly more of the vitamin than non-diabetics; while diabetics without retinopathy excrete considerably less than non-diabetics.

Procedure. All patients used in this investigation suffered from diabetes mellitus of different degrees of severity. They were drawn from the Diabetic Clinic of The Wilmer Institute of Johns Hopkins Hospital. When the diagnosis of diabetes had been established and the absence or presence of retinopathy† recorded, venous blood specimens were drawn from all subjects and allowed to clot. Sera were separated by centrifugation and were kept frozen in a deep-freeze until assay. The procedure of assay will be published in detail. Samples were identified by code numbers at Johns Hopkins Hospital and analyzed. Only after completion of the assay was the ophthalmoscopic diagnosis of the patients compared with the analytical results.

Results. Over a period of 6 months involving at least 10 different assays, blood specimens from 16 patients with retinopathy (5 males and 11 females) and from 18 patients (7 males and 11 females) were analyzed. The vit. B_{12} activity in sera expressed as micromicrograms per ml was plotted against age of the patients. It can be seen from the scatter-diagram (Fig. 1) that the B_{12} levels of the 2 groups of diabetics are distinctly different. If a line were drawn parallel to the horizontal axis at the level of 200 mg/ml, it would divide the observations clearly into 2 groups, with only one exception in the non-retinopathic group and none in the retinopathic group. The mean values of serum B_{12} levels with the standard errors of the mean are 162 ± 18 and 292 ± 24 $\mu\mu$g/ml for the non-retinopathic and retinopathic groups, respectively. The difference in the serum levels is statistically significant with $P < 0.001$.

Although the role of vit. B_{12} in metabolism

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* The authors wish to acknowledge with thanks the U. S. Public Health Service Grant No. A435.

† All diabetic patients were examined with a giant ophthalmoscope following mydriasis. Those cases were included in which the fundi could be well visualized and in which no ocular disease was present. Only the eyes with no evidence whatsoever of capillary aneurysms, exudates, or hemorrhages were classified as nonretinopathic.

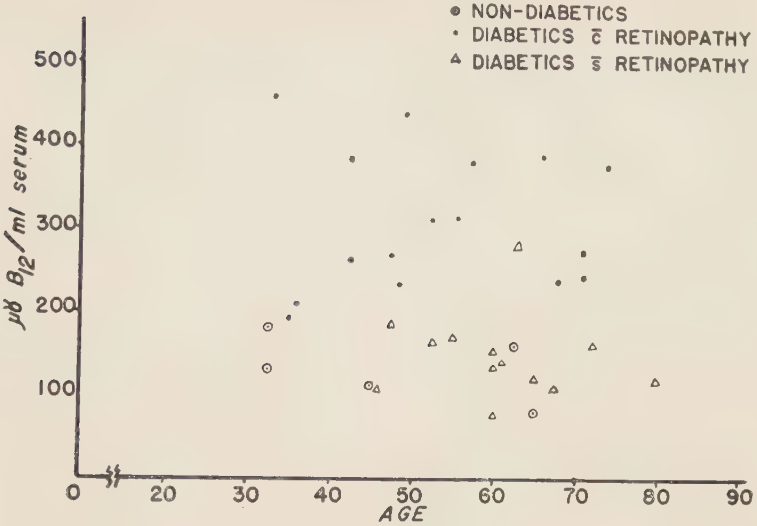

FIG. 1. Vitamin B_{12} levels in sera of diabetics with and without retinopathy.

is still not fully understood, there is a steady increase in evidence to substantiate our original hypothesis that this micro-nutrient plays an important role, directly or indirectly in carbohydrate metabolism. The data presented in this paper, coupled with those previously reported(5), make it clear that diabetics who have developed retinopathy handle vit. B_{12} somewhat differently from those who have not developed this complication. On the one hand, subjects with retinal lesions are unable to retain a normal portion of a parenteral test dose of vit. B_{12}, and on the other these same subjects have higher levels of this vitamin in the peripheral blood. The differences between these two groups of diabetics are so distinct that it is impossible to escape the conclusion that vit. B_{12} is somehow fundamentally involved in the pathogenesis of the retinopathy.

The possible role of vit. B_{12} in the correction of hyperglycemia is likewise indicated from the reports of Ling(6) in which it was found that the hyperglycemia produced following the administration of a load test of glucose to B_{12} deficient animals can be corrected by the injection of vit. B_{12}. Preliminary data from our laboratory likewise indicated that the hyperglycemia and glycosuria induced in animals by the prolonged injection of either ACTH or cortisone, or in man by the administration of cortisone alone can be corrected by the parenteral administration of vit. B_{12}. These data taken together with findings presented in this communication make it tempting to postulate that the administration of an admixture of vit. B_{12} with insulin, cortisone, or ACTH might offer certain clinical advantages over the administration of these hormones alone. However, the complexity of this disease commonly treated with these hormones makes it extremely difficult to evaluate whether co-administration of vit. B_{12} would have any effect on the prognosis.

Summary. Data are presented to demonstrate that the serum levels of vit. B_{12} activity in diabetics with or without retinopathy are widely different. Those with the retinal lesions have a much higher activity (292 ± 24) than those without retinopathy (162 ± 18). This difference is statistically significant.

The possible implication of vit. B_{12} in this disease is discussed.

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Liberation of Hydrofluoric Acid by Enzymatic Hydrolysis of Dialkyl Fluorophosphates. (21278)

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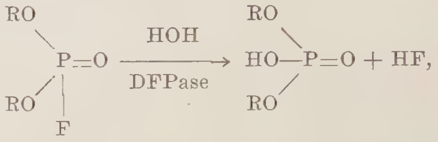
Mazur(1) first demonstrated that an enzyme in rabbit serum hydrolyzed diisopropyl fluorophosphate (DFP) to dialkyl phosphoric acid, hydrogen and fluoride ions. He also showed that enzymes (DFPases) which hydrolyzed dialkyl fluorophosphates were present in a number of rabbit and human tissues and assumed that the mode of action of these enzymes was similar to that of rabbit serum. It has been shown that the characteristics of the DFPases from rabbit serum and hog kidney differ markedly(2,3,4,5). This investigation was undertaken to determine if hog kidney DFPase was specific in hydrolyzing the P-F linkage of diisopropyl-, dibutyl- and diethyl fluorophosphates.

Materials and Methods. A purified preparation (Fraction A-2) obtained from hog kidney was used as the source of DFPase(2). Enzyme activity was determined by measurement of CO_2 evolution with the Warburg apparatus(1,2). When Mn^{++} or Mn^{++} and a cofactor were employed to activate DFPase, they were first incubated with the enzyme preparation for 15 minutes before mixing with the substrate. At different intervals, pairs of flasks, one with and one without enzyme, were removed from the water bath and their contents were immediately washed out with 30 ml of 60% ethanol and with 4 ml of chloroacetic acid/sodium hydroxide buffer (pH 3.8) into an Erlenmeyer flask. These additions stopped the enzyme action and the fluoride was titrated with thorium nitrate using sodium alizarin sulfonate as indicator(6). The solutions in the Warburg flasks usually contained less than 0.1 mg protein nitrogen which did not interfere with the fluoride estimation. When larger amounts of protein were present silver nitrate was used as the precipitating agent(1). Qualitative tests were also made for isopropyl alcohol(7) and inorganic phosphate(8). Diisopropyl fluorophosphate (DFP) was a Merck product. Dibutyl fluorophosphate (DBFP) and diethyl fluorophosphate (DEFP) were prepared from the respective chlorophosphates* by an exchange reaction with sodium fluoride(9).

Experimental. Varying amounts of the enzyme preparation, in the presence and absence of activators, were used to study the hydrolysis of DFP, DBFP and DEFP. According to the equation,



$$(RO)_2P(=O)F \xrightarrow[\text{DFPase}]{\text{HOH}} HO{-}P(=O)(RO)_2 + HF,$$

two moles of CO_2 should be liberated from the bicarbonate buffer by the hydrolysis of each

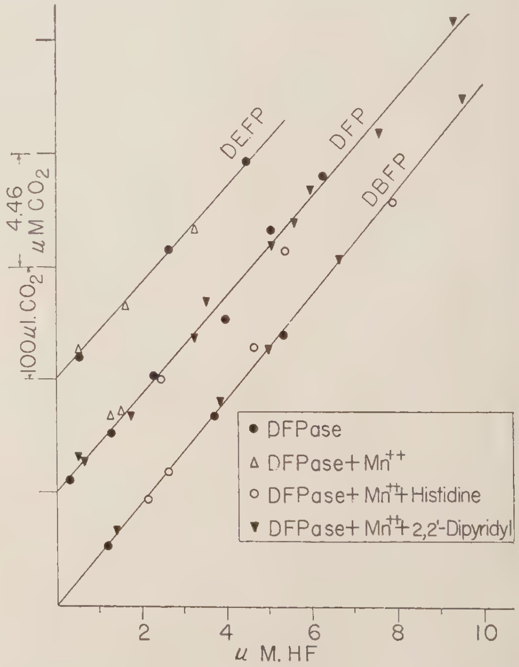


FIG. 1. Liberation of hydrofluoric acid and CO_2 during hydrolysis of diisopropyl fluorophosphate (DFP) dibutyl fluorophosphate (DBFP) and diethyl fluorophosphate (DEFP) by hog kidney DFPase.

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* The chlorophosphates were kindly donated by the Victor Chemical Co., Chicago.

mole of dialkyl fluorophosphate. The plots in Fig. 1 show the relationship between CO_2 evolved and inorganic fluoride liberated after the hydrolysis of three dialkyl fluorophosphates. According to the slopes of the lines, the CO_2/HF ratios for DFP, DEFP and DBFP are 43.5, 42.5 and 46.0, respectively, as compared to a theoretical ratio of 44.8. Within the experimental error of the methods, these data indicate that 1 mole of HF is liberated for each 2 moles of CO_2 evolved. This ratio differs from that obtained by Mazur(1) with rabbit serum (1:1.49) which may be explained by the retention of CO_2 in the presence of comparatively large amounts of plasma. These results indicate that the products of hydrolysis of dialkyl fluorophosphates by enzymes of differing characteristics are the same.

Qualitative tests for inorganic phosphate and isopropyl alcohol were negative after complete hydrolysis of DFP, both by the enzyme alone and by the enzyme in the presence of cofactors.

Cohen and Warringa(10) studied the metabolism of DFP^{32} (diisopropyl fluorophosphate labelled with P^{32}) after intramuscular injection of nontoxic doses into human subjects. They observed that 50 to 60% of the injected DFP is excreted in the urine as diisopropyl phosphoric (DIP) acid during the first 10 days. The residual DFP probably remains firmly bound as a DIP-protein complex(11). It is probable that the *in vivo* and *in vitro* hydrolyses of DFP are similar.

Summary. Evidence has been presented to show that hog kidney dialkyl-fluorophosphatase (DFPase) liberates 1 mole of hydrofluoric acid and 1 mole of dialkyl phosphoric acid in the enzymatic hydrolysis of a mole of diisopropyl fluorophosphate, diethyl fluorophosphate or dibutyl fluorophosphate.

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Heat-Stability of Antibodies in Tuberculous Sera. (21279)

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The presence of hemagglutinins and hemolysins in sera of tuberculin-negative cattle has been reported from these laboratories(1). The persistence of these antibodies in low titers over a 2- to 3-year period suggested that they might be naturally occurring or "normal" antibodies with specificities for antigens similar to those present in *Mycobacterium tuberculosis*.

Preliminary results showed that antibodies in these tuberculin-negative bovine sera were eliminated by exposure to 70°C for 10 minutes. When tuberculous bovine or human sera were similarly treated, "heat-stable" antibodies could still be detected in some samples. The possibility of greater correlation of "heat-stable" antibody with tuberculous infection prompted a further study of the heat-stability of antibodies produced in response to injections of *M. tuberculosis*, B.C.G., P.P.D., and

TABLE I. Effect of Heating 6 Normal Bovine Sera on Hemagglutination and Hemolysis with O.T.-Sensitized Cells.

Unheated serum		Heated (70°C, 10 min.) serum	
Hemagglutination titer*	Hemolytic titer	Hemagglutination titer	Hemolytic titer
16	—	—	—
8	2	—	—
16	16	2	8
4	2	—	—
8	2	—	—
16	—	—	—

* All titers expressed as the reciprocal of the dilution.

Mycobacterium phlei. Accordingly, rabbits were given each agent as indicated below and titrations of fresh and heated sera were performed at frequent intervals following injection.

Materials and methods. Technics for sensitization of erythrocytes with O.T. and procedures for hemagglutination and hemolytic tests are essentially the same as originally reported by Middlebrook and Dubos(2) and Middlebrook(3), but with a modification in the quantities of serum and cells used in the tests(4). Sera were heat-inactivated for 30 minutes at 56°C, diluted 1:2 with buffered saline and adsorbed twice (each adsorption 30 minutes at 37°C) with 1/10 volume of packed, normal sheep cells to remove heterophile antibody. Samples of sera were further heated at 70° C for 10 min. All tests were incubated at 37° C for 30 min. and examined for evidence of agglutination following light centrifugation. Similar serum-red cell mixtures containing appropriate amounts of reconstituted 1:4 guinea pig complement which had been adsorbed twice at refrigerator temperature (20 minutes/adsorption) with 1/10 volume of packed, normal sheep cells, were incubated and examined for hemolysis. The last dilution of serum showing complete lysis was recorded as the end-point of titration. Rabbits were infected by a single intravenous injection of 2 mg of *M. tuberculosis* var. *bovis* (Ravenel). Other animals were injected similarly with 2 mg of B.C.G. or *M. phlei.* All cultures were propagated on Petragnani's medium and harvested into physiologic saline. Uniformity of inocula was obtained by rotating bacterial suspensions in flasks containing glass beads. Two sources of P.P.D. were used. P.P.D. 49609 was kindly supplied by Dr. F. B. Seibert and P.P.D. X-0266-A by Parke-Davis Co. of Detroit, Mich. Animals were injected intravenously with 0.2 mg of these preparations contained in one ml of phosphate-buffered saline (pH 7.4).

Results. As seen in Table I, the exposure of cattle sera to 70°C resulted in elimination of all antibody from 5 of 6 normal sera while hemagglutinin in the sixth serum was reduced 8-fold. Similarly, agglutinins were completely eliminated from 3 of 8 tuberculous bovine sera (Table II), and from 10 of 12 samples of tuberculous human sera (Table III). Titers of the other sera in both groups were also reduced. There is no apparent correlation between unheated serum titers and amount of reduction produced by heat.

TABLE II. Effect of Heat on Hemagglutinins in 8 Tuberculous Bovine Sera.

Hemagglutination titer before heat	Hemagglutination titer after heat (70°C, 10 min.)
128	8
64	2
128	16
512	8
4	—
64	—
64	—
64	—

Serologic tests were performed on unheated and heated sera from 4 rabbits injected with

TABLE III. Effect of Heat on 12 Tuberculous Human Sera.

Unheated serum		Heated (70°C, 10 min.) serum	
Hemagglutination titer	Hemolytic titer	Hemagglutination titer	Hemolytic titer
32	4	—	—
64	—	—	—
64	—	—	—
64	64	8	32
16	—	—	—
16	—	—	—
64	—	—	—
2	—	—	—
64	8	4	2
32	—	—	—
64	4	—	—
256	8	—	—

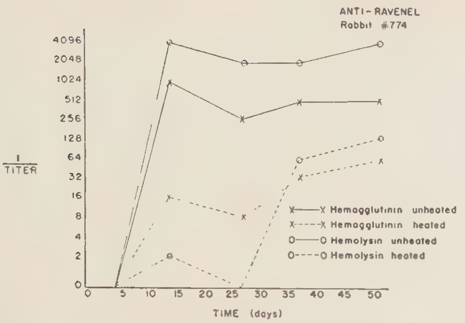

FIG. 1. Comparative antibody titers in unheated and heated tuberculous rabbit serum.

M. tuberculosis var. *bovis* (Ravenel), with samples obtained before injection and from bleedings at intervals to 50 days. Representative data are presented in Fig. 1.

Both hemagglutinins and hemolysins were present in maximal titers in unheated sera by 14th day, the hemolysin titer being higher. Hemagglutinins and hemolysins also were present in heated sera. In contrast to unheated sera, the highest titers of both heat-stable antibodies appeared later in the course of infection, occurring in maximal titers on 50th day, which was the last blood sample taken prior to death of the rabbit.

Four rabbits were injected with B.C.G. as described previously. The animals were bled periodically during the ensuing 65 days at which time they received a second injection of antigen. The results presented in Fig. 2 from a single animal are characteristic of the group. Agglutinins were demonstrable early in heated sera, but titers did not increase after 2 weeks as with tuberculous rabbit sera. Again, the hemolysin in heated sera appeared later than other antibodies and in one rabbit did not appear at all until after second injection. Following the second injection of antigen, the titers of all antibodies increased beyond the original titers.

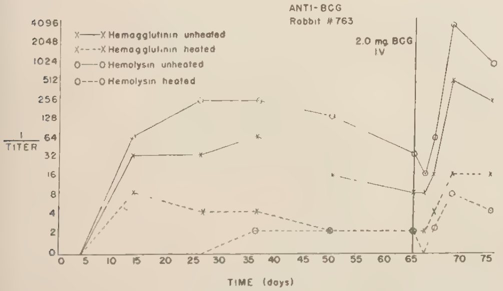

FIG. 2. Comparative antibody titers in unheated and heated sera from rabbits injected with B.C.G.

Since sera from many normal cattle contain antibodies which will react with O.T.-sensitized cells (as indicated in Table I), the suggestion was made that non-infectious mycobacteria might be responsible for the sensitization(5). The antibody response of rabbits injected with *M. phlei* was similar to results observed with sera from infected animals (Fig. 1).

Scott and Smith(6) reported stimulation of agglutinins in humans by tuberculin testing; tuberculin-positive individuals responded more actively. In a further effort to characterize the antigenic stimuli responsible for antibodies in heated sera, 8 normal rabbits were injected with P.P.D. and antibody determinations made as indicated in Fig. 3. The data are representative of the results observed with 6 of the 8 animals. No heated-serum antibodies were demonstrated until 4 days after the second P.P.D. injection. Titers were very low and disappeared 10 days later. During the same period of time titers of hemagglutinin and hemolysin in unheated sera rose to maximal levels but did not approach those observed in animals injected with Mycobacteria.

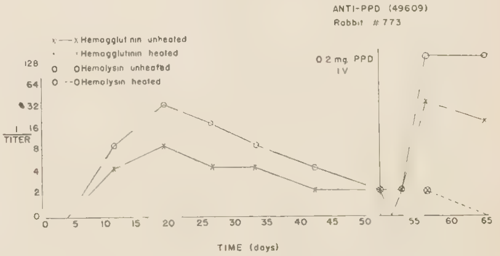

FIG. 3. Comparative antibody titers in unheated and heated sera from rabbits injected with P.P.D.

Agglutinins were present consistently in heated sera of the other 2 animals, although agglutinins in unheated sera were only demonstrated erratically. Both the heat-labile and heat-stable hemolysins appeared about the same time, but varied somewhat as to their appearance in sera from subsequent bleedings. Titers of antibodies in unheated serum rose to maximal values after the second injection of P.P.D., but differences in antibody titers in heated sera were minimal.

Discussion. The common response of rabbits to all 4 agents was primarily heat-labile antibodies. Since the earliest heat-stable antibodies were agglutinins, apparently some of the agglutinin molecules, in addition to being heat-stable, also are incapable of fixing complement to result in lysis. The heat-stable hemolysin usually appeared later, the titers of both antibodies in heated tuberculous and anti-phlei sera continued to rise.

It is evident that heat-stable antibodies are produced maximally in response to antigens present in *M. tuberculosis* (Ravenel) and *M. phlei,* less readily following injection of B.C.G., and least following P.P.D. However, both heat-stable antibodies may appear in some rabbits at the same time and quite soon after injection of P.P.D.

Follensby and Hooker(7) reported that heated equine antihemocyanin combined with antigen but lacked the capacity to precipitate optimally the same concentration of antigen demonstrated with unheated serum. They also stated that heated serum inhibited precipitation, and concluded that heating resulted in the formation of complexes of antibody globulin and serologically inert protein. Similar observations of inhibition have been made by others after studying the sera from other species or different antigen-antibody systems (8-13).

Other reasons have been presented by some of the above authors(11-13) for the effect of heat on reactivity of immune sera. The principal characteristics of heated rabbit antisera seem to be antibody which specifically combines with antigen, does not result in precipitation or agglutination and inhibits the demonstration of antibody present in unheated serum. In the present work, no inhibition could be demonstrated.

The specificity of the heat-stable agglutinins must be directed toward antigenic sites different from those required by labile hemolysin since its demonstration was not inhibited by heated serum. Further proof of the independence of the heat-stable agglutinins is the fact that their titers in tuberculous and anti-phlei sera eventually approximated their labile counterpart. The non-lytic nature of heat stable agglutinin and its distinct specificity are evidenced by the fact that an excess added to tuberculous serum failed to affect the hemolytic titer, but markedly reduced the hemolytic titer in unheated serum from normal cows.

Although practically all antibody in sera of tuberculin-negative cattle was eliminated by heat, the presence of heat-stable antibodies in the sera of only 16% of tuberculous humans and in 62.5% of the infected cattle, as well as their appearance in sera of rabbits injected with *M. phlei* seems to contradict the original premise of a greater correlation between these antibodies and tuberculosis. However, the occurrence of heat-stable antibodies in progressive tuberculosis in rabbits has been established. If the comparison with rabbits is valid, the absence of such antibodies in sera from some cases of human and bovine tuberculosis is due to lack of proper antigenic stimulus, either in quantity or kind. Thus, further study of heat-stable antibodies in experimentally infected animals and in cases of known tuberculosis is warranted to determine whether or not their continued presence may indicate a poor prognosis, or if their elimination would serve as a criterion for the effectiveness of therapy.

Summary. 1. Antibodies in the sera from a group of tuberculin-negative cattle were destroyed by heating at 70°C for 10 minutes, while heat-stable antibodies were detected in some tuberculous sera. 2. Experimental tuberculosis in rabbits was accompanied by the appearance of heat-stable agglutinins early in the course of infection, and later by heat-stable hemolysins. 3. Similar findings were observed in rabbits injected with B.C.G. and *M. phlei.* 4. Several injections of P.P.D. were required to stimulate the production of heat-stable antibodies.

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Serum Accelerator Factors and Antihemophilic Factor (AHF) in Early Phases of Clotting.* (21280)

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In recent years a number of plasma factors have been described which are required for the optimal conversion of prothrombin to thrombin. A simple, yet effective, partial separation of these substances can be achieved with $BaSO_4$ and other alkaline earth salts. At least two plasma factors, AHF and Ac-globulin, are adsorbed hardly at all with $BaSO_4$; others, particularly PTC and convertin (SPCA) are adsorbed almost completely. Prepared from plasma, the citrate eluate of the $BaSO_4$ adsorbent contains prothrombin; however, made from serum, it is free of prothrombic activity when the parent blood has been clotted with thromboplastin. The $BaSO_4$ eluate from such serum will be designated hereafter as serum accelerator factor (SA) with the understanding that it consists of at least 2, and possibly other, clotting components. A preliminary report of our findings has been published previously(1).

Materials and methods. *Blood* and *native plasma* were obtained from normal and hemophilic dogs, as described previously(2). *Platelet-poor hemophilic plasma* was prepared similarly by centrifugation at 15,000 *g* for 2 hours at 4°C in siliconed tubes. The source of the *antihemophilic factor* (*AHF*) was prothrombin-free plasma, prepared from normal oxalated dog plasma by $BaSO_4$ adsorption (40 mg/ml) and dialysis against .032% sodium citrate in 0.9% NaCl (2 hr, 4°C). Plasma from both normal and hemophilic dogs was adsorbed in a similar manner and used as the diluent in the experiments of Fig. 2. *Serum accelerator factor* (*SA*) was prepared from normal canine blood to which thromboplastin had been added. The resulting serum was adsorbed with $BaSO_4$, and the adsorbent eluted with 3.2% sodium citrate. This eluate contained no prothrombin, AHF, or Ac-globulin activity, and did not clot citrated plasma in 6 hours or fibrinogen in 10 minutes. The eluate, after dialysis against normal saline, was stored at −20°C at a concentration of 20-25 SPCA units per ml.(3). *Plasma and serum prothrombic activity* were determined by the one-stage method of Langdell, Graham and Brinkhous(4), after the samples had been diluted with prothrombin-free normal or hemophilic plasma. *Plasma and serum prothrombin concentration* were determined by the Iowa 2-stage method(5), modified by the addition of Factor V(6) to the diluting fluid.

Results. The effect of the addition of SA was determined in several types of clotting systems.

The reaction of *hemophilic whole blood* is shown in Table I, Tests 2-5. The addition of SA caused the clotting time to be reduced to the normal range of 5-8 minutes, but did not alter the defective prothrombin utilization (Test 3). When SA and AHF were added together, both clotting time and prothrombin utilization were more rapid than in normal blood (cp. Tests 1 and 5). The prothrombin

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TABLE I. Effect of Serum Accelerator (SA) and Antihemophilic Factor (AHF) on Clotting of Hemophilic Whole Blood and Hemophilic Platelet-Poor Plasma.

Test No.	Coagulant added	Clotting time, min.	Residual prothrombin, %* Min. after drawing blood 15	30	60	120
	Normal whole blood (1 ml)					
1	0.9% NaCl (.1 ml)	7½	61	15	—	—
	Hemophilic whole blood (1 ml)					
2	0.9% NaCl (.1 ml)	30	100	100	100	100
3	SA (20 u/ml, .1 ml)	6	105	100	90	100
4	AHF (.1 ml normal pl)	8	78	10	—	—
5	SA + AHF (3 + 4)	4	22	10	—	—
	Hemophilic platelet-poor plasma (0.5 ml)					
6	0.9% NaCl (.1 ml)	84	—	90	—	92
7	SA (25 u/ml, .1 ml)	8	—	—	85	72
8	AHF (.1 ml normal pl)	24	—	85	85	72
9	SA + AHF (7 + 8)	8	—	78	78	74
10	AHF + platelets (.1 ml normal pl + 2×10^8 platelets)		10	—	—	—

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* In all experiments the 100% prothrombin control was a sample of blood (1 ml) or plasma (0.5 ml), citrated with 0.15 ml of 3.2% sodium citrate. Residual prothrombin was determined by the 2-stage method.

utilization curves indicated that the prothrombin "half-life", time required for conversion of 50% of the prothrombin, was 18¾ minutes in normal blood (Test 1), 21 minutes in hemophilic blood with AHF alone (Test 4) and 10 minutes with both SA and AHF (Test 5).

Hemophilic platelet-poor plasma reacted quite differently (Table I, Tests 6-10). After addition of SA, either alone or with AHF, the clotting time became nearly normal, but utilization of prothrombin remained impaired. By contrast, addition of AHF and platelets (Test 10) corrected completely the defect in prothrombin utilization.

The results with *normal native plasma* containing platelets are shown in Fig. 1. On addition of SA, prothrombin was utilized more quickly than in the control plasma. This change is similar to that seen when comparing Tests 1 and 5 of Table I. The prothrombin "half-life", interpolated from Fig. 1, was 24 minutes for the control, 8½ minutes with the lesser quantity of SA and 5 minutes with the greater. The latent period, the period prior to detectible prothrombin loss(7), was 12-16 minutes in the control, 3½-5 minutes with the smaller SA addition and 0-2 minutes with the larger quantity.

The *prothrombic hyperactivity of hemophilic serum* was described in an earlier publication(4) and has been attributed to the presence of serum accelerators plus a high residual prothrombin concentration. The experiments shown in Fig. 2 duplicate and extend our previous findings. Repeating the earlier experiments, but using $BaSO_4$-treated hemophilic plasma in addition to similarly treated normal plasma as a diluent and source of fibrinogen, we found that the usual prothrombic hyperactivity could not be elicited in the absence of AHF.

Discussion. Several interesting facts emerge from the experiments described above. The SA corrects the prolonged clotting time of hemophilic blood without altering the fundamental defect, the block in prothrombin utilization. A somewhat similar experiment has been reported by Deutsch and Schaden(8). This finding implies that assays for antihemophilic activity should be regarded with skepticism when based on a clotting time type of procedure and when the serum factors have not been removed. It is apparent also from Table I that, even with AHF assays of the prothrombin utilization type, the serum factors may cause serious error, *provided* AHF is also present.

As shown in Fig. 1, the clotting time may be shortened markedly and the latent period almost abolished without significant effect on the rate of prothrombin utilization. The dissociation of latent period and reaction rate in

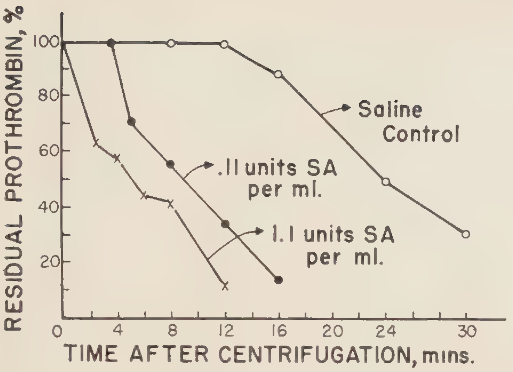

FIG. 1. Prothrombin utilization in native plasma from a normal dog. Eight experiments were performed, each similar to the one shown above. Platelet counts ranged from 5600 to 173600/mm^3, but results were qualitatively similar in all. In the experiment above, native plasma (9800 platelets/mm^3) was prepared from a normal dog by low-speed centrifugation in siliconed glassware at 4°C. Into each plain glass test tube at 28°C was placed 0.5 ml of the plasma. Also present in each was 0.1 ml of either 0.9% NaCl or SA. The SA concentrations, shown on the figure, are expressed in SPCA units per ml of reaction mixture. The clotting time of the saline series was 7½ min., with the smaller quantity of SA 2¾ min., with the larger quantity of SA 2¼ min.

both hemophilic and normal systems is of interest. It had been assumed that the length of the latent period in prothrombin conversion always varied inversely with the reaction rate as in many other biochemical reactions. More detailed experiments are indicated, but it would appear that the eluate operates very early in prothrombin conversion and lacks significant acceleratory effect once the reaction is underway. These results should not be interpreted as meaning that the eluate factors are inactive once thrombin appears.

Previous work had shown that AHF markedly affected the *rate* of prothrombin utilization(9). It is possible that SA and AHF operate sequentially during prothrombin conversion, SA acting earlier. This is contrary to the interpretation of Owren(10), if it is assumed that SA is equivalent to convertin. He believes AHF to act initially, and convertin, a serum factor, to be active later. Our suggestion is consistent, however, with the interpretation of Biggs, Douglas and Macfarlane(11), if one assumes that the latent period shortening by SA is the expression of its PTC rather than its convertin moiety. Our experiments do not show which specific factor of the eluate is responsible for the latent period effect. This answer awaits further chemical fractionation.

The necessity of platelets for prothrombin utilization is reemphasized by the data of Table I. From some of the literature (10,11), one would assume that platelets are not required once prothrombin begins disappearing. Our experiments suggest, on the contrary, that platelets are required throughout the period of prothrombin utilization, at least in blood and plasma. It is possible that the platelet, in combination with some reaction product perhaps, constitutes the "autocatalytic agent."

The basis of the hyperactive prothrombin time of serum shown in Fig. 2 (values >100%), is clarified by the experiments of Table I and Fig. 1. These data suggest that the increased "speed" of clotting is due primarily to a decreased latent period. This same effect may be produced by adding the eluate to normal plasma. The fact that this

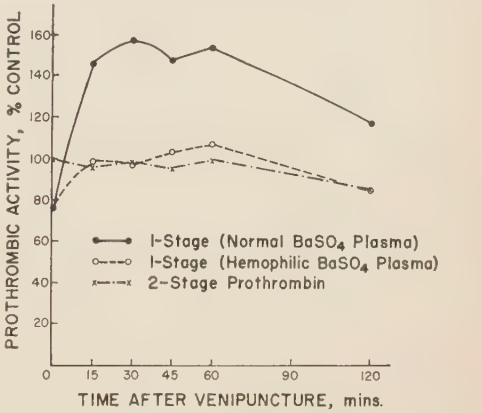

FIG. 2. One-stage and 2-stage prothrombin determinations in clotting hemophilic dog blood. At intervals 0.3 ml of 3.2% citrate was added to 2 ml of hemophilic blood clotting at 28°C, and the blood centrifuged immediately at 5000 *g* for 3 min. The serum was tested within 5 min. of citration by the one-stage technic. The actual reaction mixtures in the one-stage determination consisted of: 0.1 ml serum diluted 1-10 with $BaSO_4$ plasma + 0.1 ml Difco brain thromboplastin + 0.1 ml 0.02 M $CaCl_2$. Per cent prothrombic activity for each sample was determined by interpolating clotting time values on a dilution curve prepared by diluting the 100% control plasma with $BaSO_4$-adsorbed normal plasma. This control plasma had sat 35-40 min. at room temperature until its clotting time had reached a minimum. These minima varied from 11.5 to 14 sec. in different experiments. Two-stage determinations of prothrombin concentration were carried out later.

shortening in the prothrombin time, equivalent to a 50% increase in prothrombin, is accomplished by an eluate without prothrombin and containing an infinitesimal amount of thrombin, suggests that the substances responsible are accessory to the main reaction and not direct contributors to the thrombin pool.

One might speculate endlessly over the synergism of SA and AHF shown in Fig. 2. We were surprised to find that supernormal prothrombic activity could not be obtained without AHF, despite the use of a potent brain thromboplastin. Possibly the simplest and least controversial inference which might be drawn from the experiments of Fig. 2 is that AHF, under certain conditions, is merely another of the numerous variables in the prothrombin time test.

Summary. 1. An eluate from $BaSO_4$-adsorption of normal serum is shown to have a marked effect on the clotting time in hemophilia without altering the defect in prothrombin utilization. 2. The eluate is shown to operate chiefly by reducing the latent period of prothrombin utilization. 3. The effect of this eluate on AHF assays is discussed. 4. The necessity of platelets for prothrombin conversion is demonstrated again under different experimental conditions. 5. AHF is shown to be required for the demonstration of maximal prothrombic activity in the prothrombin time test.

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Simplified Method for Determination of Total Adrenal Cholesterol.* (21281)

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(Introduced by E. M. Landis.)

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The determination of total adrenal cholesterol by the method of Schoenheimer and Sperry(1) or modifications thereof has been a cumbersome and time-consuming procedure. The publication of a simple and rapid direct method for the determination of serum cholesterol by Zlatkis, Zak, and Boyle(2) prompted us to adapt their procedure to the determination of adrenal cholesterol. The modified Zlatkis, Zak, and Boyle procedure was subjected to comparison with that of Sperry and Webb(3) with good agreement between the 2 methods.

Methods. Reagents. With a few minor modifications, the reagents and following procedures are those previously described by Zlatkis, Zak, and Boyle(2) for the determination of total serum cholesterol. 1. *Standard cholesterol solution*: 0.2 mg pure, dry, ash-free cholesterol/ml of glacial acetic acid (C.P. 99.7%). 2. *Ferric chloride sol.*: Dissolve 10 g of ferric chloride, reagent grade in 100 ml glacial acetic acid. 3. *Color reagent*: Dilute 1.0 ml of ferric chloride solution to 100 ml with C.P. concentrated sulfuric acid.

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Procedure. A. Standard curve: .1, .2, .3, .4, and .5 ml of the standard cholesterol solution are pipetted into matched colorimeter tubes (19 x 150 mm) and diluted to 3.5 ml with glacial acetic acid. The blank contains 3.5 ml of glacial acetic acid. 2.5 ml of the color reagent are pipetted into each tube, allowing the color reagent to slowly flow down the side of the tube. The resulting 2 layers are then mixed rapidly by applying a brisk circular motion to the tube. This process is a critical step in the procedure and should be executed uniformly in all samples. When the tubes have reached room temperature, the absorbency is measured in a spectrophotometer at 560 mμ. B. Determination of total adrenal cholesterol: 1. Place cleaned and weighed adrenal in a McShan homogenizer(4) and homogenize the gland. 2. Add 10 ml of C.P. glacial acetic acid and mix thoroughly. 3. Filter the homogenate through Munktell's No. 00 filter paper. 4. Pipette 0.5 ml of filtrate into a 19 x 150 mm colorimeter tube. 5. Add 3.0 ml of glacial acetic acid and mix. 6. Add 2.5 ml of color reagent and mix as described above and allow to come to room temperature. 7. Read at 560 mμ as above. 8. Cholesterol content of aliquot is determined from standard curve. 9. Calculation: [Value from standard curve x 20 x 100]/[adrenal weight(mg)] = mg cholesterol/100 mg adrenal.

Experimental. In order to compare adrenal cholesterol values obtained by the direct method of Zlatkis, Zak, and Boyle with current methods based on the Liebermann-Burchard reaction, the following procedures were followed. It was first ascertained that there is no statistically significant difference between the total cholesterol concentration of the right and left adrenal glands from adult female rats of the Sprague-Dawley strain (Table I). A group of 28 normal, adult female rats of the same strain were then sacrificed by ether inhalation and the adrenal cholesterol concentration of the left adrenal was determined by the method of Zlatkis, Zak, and Boyle, while that of the right adrenal by the procedure of Sperry and Webb(3) as adapted for adrenal cholesterol by Agate(5). The results are shown in Table II, Exp. 1. It is seen that the 2 methods give identical values. To determine whether this agreement between the 2 methods would still hold following alterations in adrenal cholesterol concentration by experimental means, 3 groups of adult female rats were studied as above. One group of 6 animals served as control, a group of 14 animals was hypophysectomized and adrenal cholesterols determined by the 2 methods 7 days following operation and a third group of 10 rats was placed in a cold room at 5°C and adrenal cholesterol content was assayed following a 24-hour exposure to this temperature. The results are given in Table II, Exp. 2. The differences between the adrenal cholesterol values obtained by the 2 methods are not statistically significant. It will be noted, however, that the results of the Zlatkis, Zak, and Boyle method are somewhat higher in most instances than those of the Sperry-Webb procedure. This is in agreement with the findings of Zlatkis, Zak, and Boyle(2) for serum cholesterol. These authors attribute this slight difference to the necessity of employing several steps in methods based on the Liebermann-Burchard reaction as compared to the few steps required by their direct method with concomitant reduction in the possibility of cholesterol loss during the course of the latter analysis.

TABLE I. Total Cholesterol Concentration of Right and Left Adrenal Glands in Adult Female Rats. (Method of Zlatkis, Zak and Boyle.)

Treatment	No. of rats	Left adrenal	Right adrenal	P
None	12	5.40 ± .24*	5.56 ± .26	>.1

* Mean ± stand. error of mean.

TABLE II. Cholesterol Concentrations in Left Adrenal (Zlatkis, Zak and Boyle) vs. That in Right Adrenal (Sperry-Webb) in Normal, Hypophysectomized and Cold Stressed Rats.

Exp.	Treatment	No. of rats	Left adrenal (ZZB)	Right adrenal (SW)	P
I	None	28	5.10 ± .17*	5.12 ± .22	>.1
II	”	6	4.78 ± .35	4.17 ± .38	”
	Hypox.	14	10.68 ± .31	10.12 ± .35	”
	Cold	10	2.44 ± .18	2.18 ± .16	”

* Mean ± stand. error of mean.

Conclusions. The method of Zlatkis, Zak, and Boyle for the direct determination of serum cholesterol has been adapted to the determination of total adrenal cholesterol. The values thus obtained do not differ significantly from those obtained by the method of Sperry and Webb in normal, hypophysectomized and cold stressed animals. The time required to analyze 10 samples and 5 standards by the Zlatkis, Zak, and Boyle procedure requires less than one hour compared to the 2 days usually necessary when methods based on the Liebermann-Burchard reaction are utilized.

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Hyaluronic Acid Formation by *Streptococcus pyogenes*.* (21282)

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Previous work(1) with 4 encapsulated strains of *S. pyogenes* demonstrated that these microorganisms form from 30 to 50 mg of hyaluronic acid per 100 ml of culture when grown for 12 hours in 1% glucose infusion broth. When galactose is substituted for glucose as the carbon source only 5 to 10 mg of the capsular polysaccharide can be detected per 100 ml of culture in the same period of time. That the substance found in such cultures is hyaluronic acid is indicated by our observation that it is susceptible to hydrolysis by both streptococcal and testicular hyaluronidases and that it precipitates under the same conditions as does the material from the glucose-containing cultures.

It has been reported(2,3) that the intact carbon skeleton of glucose is incorporated into the structure of hyaluronic acid by *S. pyogenes* in glucose-containing media. No data are available on the mechanism of capsule synthesis in the presence of galactose but certain factors which influence the production of hyaluronic acid in both glucose and galactose broth cultures of *S. pyogenes,* strain S23, will be reported here.

Methods. Organism S. pyogenes, strain S23, a type 14 mucoid organism, was used throughout. *Culture Media* Two and one-half per cent heart infusion broth (Difco) supplemented with 0.5% neopeptone (Difco) and 1.0% sugar was employed. The medium was adjusted to pH 7.5, diluted to 98% of final volume and autoclaved. Sufficient sterile 10% $NaHCO_3$ was then added to bring the medium to volume. The final pH, before inoculation, was approximately 7.8. *Cultivation* Culture stocks were maintained on coagulated whole egg slants with transfers at monthly intervals. Blood agar plates were streaked periodically to detect dissociation. This strain displayed a uniform capacity to synthesize hyaluronic acid when maintained under these conditions. Inocula, consisting of the centrifuged cells from 17 hour cultures in the standard medium modified to contain 0.2% glucose, were used in the hyaluronic acid formation studies. The volume of each inoculum culture was one-tenth that of the medium to be inoculated. Incubation of all cultures was at 37° C. *Quantitative Determinations* For the estimation of bacterial nitrogen and hyaluronic acid, two 5 ml aliquots of each culture were removed and centrifuged. The supernates were carefully withdrawn, pooled, and the individual cell sediments washed once with 5 ml of saline. Nitrogen determinations were performed as previously reported(1). The pooled supernates were heated in a water bath at 60° C for 45 minutes to remove trace amounts of

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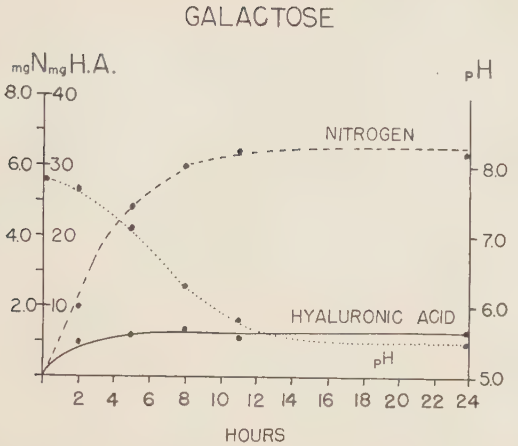

FIG. 1. Growth, pH, and hyaluronic acid curves of *S. pyogenes* strain S23 in 1% galactose infusion broth. Values per 100 ml of medium.

hyaluronidase activity. The pH was then adjusted to 6.0 and 0.1 ml of 1% merthiolate added as a preservative. Such supernates, when stored at 4° C, gave reproducible hyaluronic acid values for at least 6 weeks. Hyaluronic acid was measured by the procedure previously described(4).

Experimental. Rate of hyaluronic acid formation in glucose and galactose broths. To determine the approximate rate of formation of the capsular material successive samples, during a 24 hour period, were analyzed for hyaluronic acid and bacterial nitrogen. The hydrogen ion concentration of the cultures was also measured at each sampling period. The curves thus obtained are plotted in Fig. 1 and 2. As shown in the figures, growth is more vigorous in glucose broth and is accompanied by an earlier fall in pH. In the glucose cultures an inhibitory hydrogen ion concentration is reached after about 5 hours, whereas in galactose broth some growth is still detectable at 8 to 10 hours. The rate of hyaluronic acid synthesis in glucose broth is maximal as the pH falls from about 7.0 to below 6.0, and continues at a slower rate until growth ceases due to the accumulation of acid. In galactose broth, polysaccharide formation is rapid only during the first 2 hours, before the pH has fallen to 7.0. After 2 hours, when the pH is below 7.0, there is little or no synthesis of capsular material.

Hyaluronidase formation. Pike(5) has reported that some streptococcal strains which synthesize capsular material during the early hours of growth in broth media, later destroy soluble capsular substance as a consequence of hyaluronidase formation. Likewise Sallman (6) has shown that many *S. pyogenes* strains may produce at least small amounts of the enzyme, if grown under suitable conditions. Thus, the low concentrations of hyaluronic acid encountered in galactose broth cultures could result from the activity of hyaluronidase elaborated by the organisms during growth. Hyaluronidase activity was measured as follows: At various intervals during the first 24 hours of growth, 10 ml samples were withdrawn, centrifuged, and the supernates adjusted to pH 6.0. Merthiolate was added to give a final concentration of 0.01%. This concentration has been shown not to inhibit hyaluronidase activity(7). The sample was then divided into 2 equal parts. One was heated at 60° C in a water bath for one hour to inactivate the enzyme. The heated and unheated samples were then incubated in rubber-stoppered tubes at 37° C for 5 days, after which time hyaluronic acid determinations were made. The results are presented in Table I. It appears unlikely, on the basis of these results, that the amount of enzyme produced by this strain in galactose broth is sufficient to account for the reduced capsular polysaccharide levels found in such cultures after 24 hours of incubation.

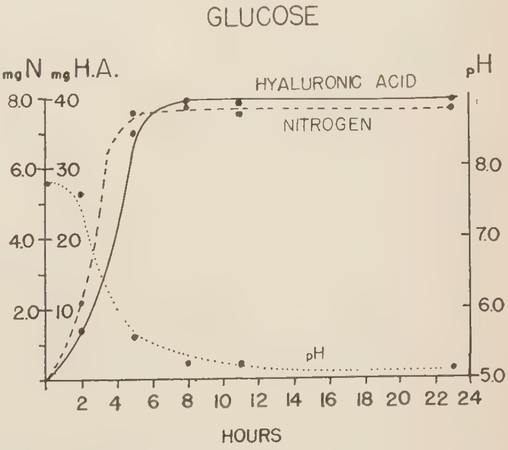

FIG. 2. Growth, pH, and hyaluronic acid curves of *S. pyogenes* strain S23 in 1% glucose infusion broth. Values per 100 ml of medium.

TABLE I. Hyaluronidase Production in Glucose and Galactose Broths.

	Hyaluronic acid (mg/100 ml)			
	Glucose		Galactose	
Time of sampling, hr	Heated supernate	Unheated supernate	Heated supernate	Unheated supernate
2	6	6	5	5
5	32	32	6	2
8	37	36	7	2
11	37	37	6	1
23	37	36	6	1

Effect of pH on hyaluronic acid formation in galactose broth. Inspection of Fig. 1 and 2 shows that hyaluronic acid synthesis in galactose broth occurs almost exclusively before the reaction of the medium has dropped below pH 7.0. The utilization of galactose for hyaluronic acid production might therefore involve at least one pH dependent metabolic step which occurs readily only at an alkaline reaction. To examine this hypothesis heavily inoculated cultures of strain S23 were grown for 6 to 8 hours in mechanically stirred galactose broth at either pH 7.8-8.0 or pH 6.6-6.8. The hydrogen ion concentration was determined with a glass electrode at 15 to 30 minute intervals and pH changes in the alkaline cultures controlled by the addition of sterile 6 N NaOH. Large amounts (35 mg/100 ml) of polysaccharide were formed in control glucose broth cultures at pH 7.8-8.0. No quantitative differences in the production of polysaccharide on galactose were found at the two pH ranges tested although growth was satisfactory. It appears therefore that hydrogen ion concentration is not an important factor controlling the formation of polysaccharide in galactose broth under our conditions.

Use of endogenous cell substance; occurrence of dissociation. If the hyaluronic acid synthesis observed in galactose broth cultures results from utilization of internal reserves of carbohydrate then the exhaustion of such reserves should result in cessation of polysaccharide synthesis. To examine this possibility, strain S23 was transferred concurrently at 24 hour intervals through 7 consecutive cultures in 0.2% glucose and 0.2% galactose broths. One milliliter inocula were added to 49 ml of media at each transfer, and blood agar plates were streaked to detect dissociation. After the seventh transfer, the galactose- and glucose-propagated organisms were each inoculated into 1% glucose and 1% galactose broths for hyaluronic acid and bacterial nitrogen determinations. No quantitative differences were observed in the formation of hyaluronic acid in galactose broth between the first and seventh transfer. In addition it was found that cells kept in galactose broth continuously for one week were still able to form capsular substance in either medium. It appears unlikely on the basis of these observations, that the polysaccharide found in galactose culture supernates is produced from reserve cellular material. Appreciable dissociation to the glossy phase did not occur during transfer in either medium.

Utilization of an unidentified medium component. Use of E. coli-fermented broth. In galactose broth cultures hyaluronic acid may be synthesized from some unidentified component or components present in the medium in limited concentration. That the reducing sugar of the heart infusion is not involved was demonstrated by the use of infusion broth exhaustively fermented with *E. coli*. Strain S23 either does not grow or grows only to a very limited extent in such a medium unless sugar is added. When 1% of galactose is supplied, growth and hyaluronic acid production occur in the usual manner. In a typical experiment the medium, without added sugar, was fermented with *E. coli* for 48 hours at 37° C and residual reducing sugar determined as glucose by the method of Nelson(8). The reducing sugar of the medium was reduced from 20 mg/100 ml to 2.3 mg/100 ml by this procedure. The nature of the residual carbohydrate has not been determined. On a quantitative basis this small amount of carbohydrate could not account for the amount of capsule produced. Attempts to re-inoculate *S. pyogenes* into fermented galactose containing broth, even after neutralization and supplementation with galactose, always resulted in failure of the organism to grow.

Modification of the medium. The galactose medium was modified in a number of ways in an attempt to establish a correlation between hyaluronic acid production and one of the medium constituents. Protein hydrolysate was

TABLE II.
Effect of Modifications of Galactose Broth Medium upon Hyaluronic Acid Formation.

Heart infusion (%)	Neopeptone (%)	Hydrolysate* (%)	Galactose (%)	Hyaluronic acid (mg/100 ml)	Bacterial nitrogen (mg/100 ml)	$\frac{\text{H. acid}}{\text{N}}$ ratio
			Experiment 1			
4.0		.5	1.0	8	6.7	1.2
2.0		.5	1.0	4	5.0	.8
.5		2.0	1.0	2	4.6	.4
.2		2.0	1.0	1	2.5	.4
.1		2.0	1.0	0	2.1	—
2.5	.5		.1	2	3.0	.7
2.5	.5		1.0	6	5.4	1.1
2.5	.5		3.0	10	7.4	1.4
2.5	.5		4.0	9	6.2	1.5
			Experiment 2			
.2		2.0	4.0	5	1.8	2.8
.8		2.0	4.0	11	3.1	3.5
1.6		2.0	4.0	13	5.3	2.5
4.0		2.0	1.6	11	3.1	3.5
4.0		2.0	.8	11	6.0	1.8
4.0		2.0	.2	5	3.9	1.3

* Tryptic digest of casein.

used because of its relative freedom from carbohydrate. As indicated in Table II, no clear-cut relationship between any of the medium constituents and hyaluronic acid synthesis could be found. One could infer from these data that for maximal growth and capsule formation, heart infusion and a utilizable source of energy such as galactose must both be present in fairly high concentration.

Discussion. The experimental data reported here favors, in general, the idea that galactose is not utilized for capsule synthesis. The fact that increased concentrations of heart infusion stimulate hyaluronic acid formation supports the possibility that a constituent of the infusion other than glucose may be the source of raw material for the synthesis of the polysaccharide. We have at present no indication of the nature of this constituent.

Crowley(9,10) has demonstrated amylase and "depolymerase" activity in broth cultures of certain serologic types of Group A streptococci. Various substrates were hydrolyzed, including starch, inulin, citrus pectin, bacterial dextrin, and glycogen. In general there was a direct correlation between these activities and hyaluronidase production. We have found slight hyaluronidase activity in supernates of galactose but not glucose broth cultures of strain S23. It is possible that some amylase and/or depolymerase activity may occur during growth in this medium, leading to degradation of infusion carbohydrate of high molecular weight with the release of smaller units utilizable for capsule synthesis.

Summary. 1. *S. pyogenes,* strain S23, grows rapidly in glucose broth, forms large amounts of hyaluronic acid throughout the growth period and induces a rapid fall in the pH of the medium. In galactose broth growth is slower, polysaccharide formation is diminished, occurring only during the first 2 or 3 hours of growth and the reaction of the medium remains above pH 7.0 for a much longer period of time. 2. While no hyaluronidase could be detected in glucose culture supernates, a small amount of activity was detected in galactose supernates. It was not of sufficient magnitude to account for the low levels of hyaluronic acid in galactose cultures. 3. Maintenance of galactose broth cultures at pH 7.8-8.0 did not increase hyaluronic acid formation. 4. It has been shown that neither endogenous cell substance nor infusion reducing sugar serve as raw materials for capsule synthesis in galactose broth. 5. A direct correlation between hyaluronic acid formation and galactose concentration could not be established. 6. From the data obtained, it seems likely that galactose is not directly involved in hyaluronic acid

formation, but that an unidentified component of the infusion is utilized for this purpose.

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Ethionine-Induced Depression of Plasma Antihemophilic Globulin in the Rat. (21283)

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To date there have been few studies relevant to the origin of antihemophilic globulin (AHF). Boldyreff(1) reported some work with dogs from which he concluded that the pancreas produced AHF. In these studies, either pancreatic fistula or complete pancreatectomy was followed by a prolongation of the whole blood coagulation time; however, no demonstration of the nature of the clotting defect was made. Graham and associates(2) produced acute liver injury in dogs by chloroform intoxication. In these animals AHF was well preserved despite a degree of liver damage which caused almost complete depletion of prothrombin and fibrinogen. Moreover, it was found that dicumarol was unable to depress AHF even at a dosage sufficient to produce severe hypoprothrombinemia. These studies would seem to exclude the liver as the source of AHF, in contrast to its role in the synthesis of most clotting factors. This same group of investigators showed that AHF was unaffected by heavy total body X-irradiation(3), indicating that the cells which produce AHF must be relatively radio-resistant.

Ethionine, the ethyl analog of methionine, is a metabolic blocking agent which interferes with the synthesis of certain proteins and produces striking histological changes in some tissues(4-6). In the studies to be reported, it was found that this drug produced a profound depression of plasma AHF levels in rats. The tissues which exhibited damage were interpreted as suggested sites for further and more definitive investigation of a possible role in AHF production.

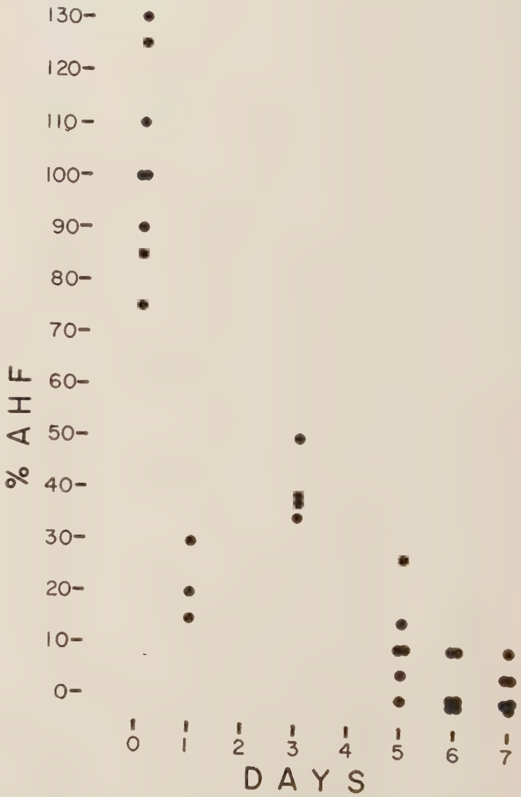

FIG. 1. AHF activity in plasma of rats treated with ethionine. Each dot represents one animal.

Methods and materials. Male albino rats

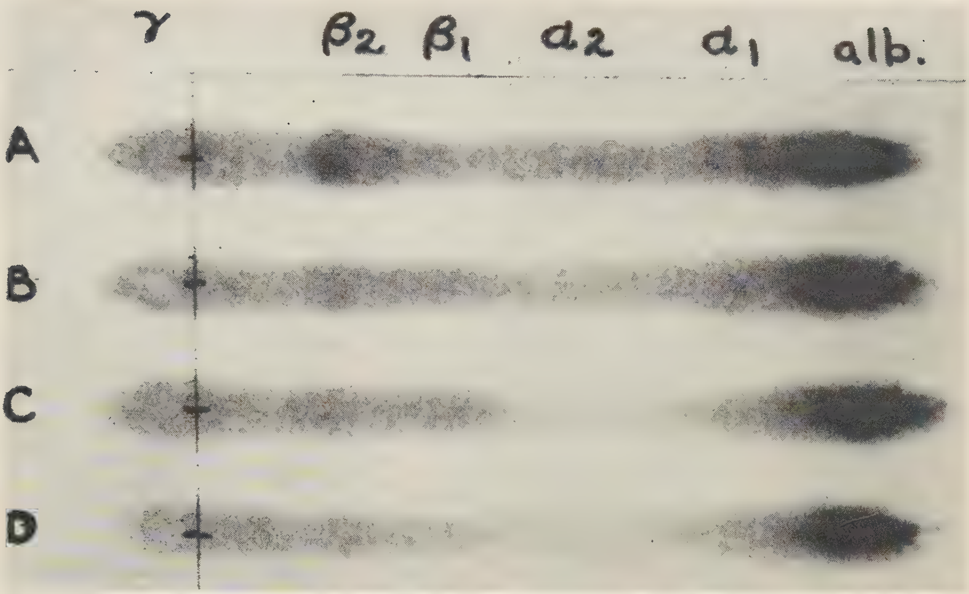

FIG. 2. Paper electrophoretic patterns of rat sera. A. Normal control. B-D. Animals after 3 days of ethionine injections.

of the Slonaker strain, weighing 175-225 g were used. Experimental animals were given intraperitoneal injections of ethionine.* The daily dose was 250 mg: 10 cc of a 2.5% solution of crystalline ethionine in distilled water. *Blood specimens* were obtained either from the internal jugular vein or directly from the heart, depending on whether subsequent specimens were required. In either case, the site of puncture was surgically exposed, and blood was obtained without contamination by tissue fluid. As the anticoagulant, one part of 0.1 molar potassium oxalate was used for nine parts of blood. Generally one cc of blood was removed. *AHF* was assayed in a manner similar to that used by Spaet and Kinsell(7). In this method, the activity of the test plasma is measured by its ability to improve prothrombin consumption in the blood of a known hemophiliac. Several different hemophiliacs provided assay blood for this study, and a curve was constructed for each patient in which serial dilutions of plasma obtained from a pool of five normal rats were tested against each patient's blood. The plasmas of the experimental and control animals were diluted 2:3 with saline and 0.1 cc of these dilutions was added to 1.9 cc of hemophilic blood, giving a final ratio of 2 parts test plasma to 98 parts hemophilic blood. The serum prothrombin was determined 4 hours after mixing, and the AHF content of the specimen estimated by comparison with the control curve. True prothrombin and proconvertin were measured by the method of Owren and Aas(8), and labile factor was assayed by the Quick technic(9). Tissues were fixed in Bouin's solution, and stained with hematoxylin and eosin. Liver fat was demonstrated by Sudan IV staining of frozen sections. Bone marrow specimens were made by smearing fresh material obtained from the femur. Marrow slides were stained with Wright's and counterstained with Giemsa. Paper electrophoresis of serum proteins was accomplished by a modification of the method of Kunkel and Tiselius(10).

Results. Fig. 1 shows the effect of ethionine on plasma AHF. A significant reduction in activity is seen after the first dose of the drug, and this depressed activity is maintained so long as ethionine administration is continued. When the drug was discontinued, there was a gradual restoration of plasma AHF starting within three days. Ethionine did not inactivate rat AHF *in vitro,* as shown by the follow-

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* Purchased from U. S. Industrial Chemicals Co., San Francisco.

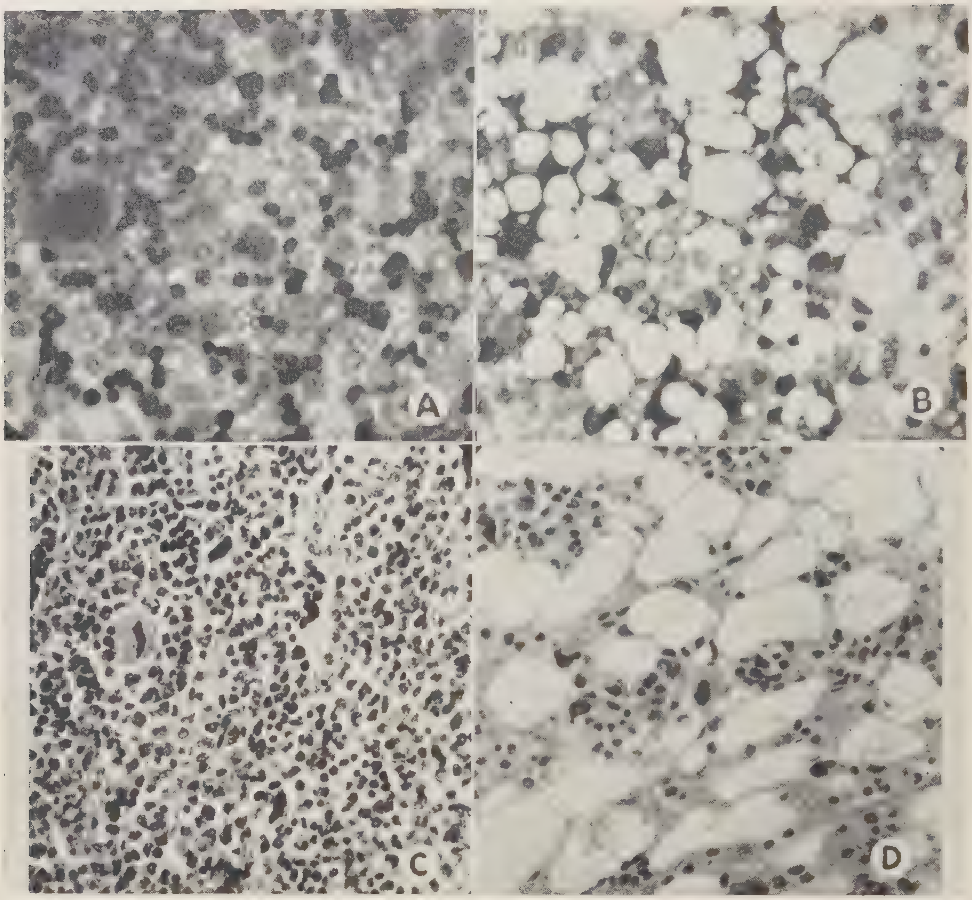

FIG. 3. Effect of ethionine on bone marrow. A. Normal rat, smear preparation, Wright-Giemsa stain. B. Ethionine-treated rat, same technic. C. Normal rat, marrow section, hematoxylin-eosin stain. D. Ethionine-treated rat, same technic as C.

ing experiment: serial dilutions of ethionine were added to oxalated rat plasma, and these mixtures were incubated at 37° C for thirty minutes. No plasma so treated showed any loss of AHF activity. These findings indicate that the ethionine-induced depression of AHF represented a true metabolic effect.

In the ethionine treated animals there was a prompt reduction of true prothrombin, proconvertin, and labile factor, confirming the studies of Witte(11). However, fibrinogen was little affected. As with AHF these coagulation changes persisted as long as the drug was continued. Electrophoretic patterns of the experimental animals showed consistent changes starting on the second day of treatment, as seen in Fig. 2. Alpha and beta globulins were markedly depressed, but albumin and gamma globulin were unaffected, even after ten days of treatment. These results confirm the selectivity of ethionine action on protein synthesis.

The following results were obtained from histological surveys of the tissues obtained from ethionine treated animals: in contrast to previous reports(12,13), no changes were seen in our animals following a single dose of the drug despite the striking changes found in the clotting factors. It would appear that the male Slonaker rats used were less susceptible to tissue damage than the males of other species used by other investigators. After three days of high dosage injections the pancreas did show typical changes consisting of loss of basophilia, zymogen granules, and acinar structure. The liver displayed nuclear changes and increased fat deposition. A hitherto undescribed finding was that of severe bone marrow depression. The marrow changes are shown in Fig. 3, and consist of diminished cellularity with replacement of the normal cells by fat, hemorrhagic areas, and vacuolization of many of the reticulo-endothelial cells. Sections of the kidney, gut, spleen, muscle, salivary glands, and lymph nodes showed no significant changes. Further evidence of the resistance of the Slonaker strain to ethionine was seen in the prolonged survival of these animals on these huge daily doses of the drug. The treated animals lived from 6 to 12 days despite a dose reported fatal to other strains within 72 hours.

None of the control animals had any consistent clotting disorder or tissue changes.

Because of the damage inflicted by ethionine on the pancreas and liver, further studies were performed on these organs. Attempts were made to destroy the pancreas in the rat by total surgical extirpation and by ligation of the common bile duct. Since neither of these measures gave satisfactory pancreatic destruction, use was made of dogs with complete surgical pancreatectomy. These animals were 2 months postoperative, and had been given only insulin treatment for 2 weeks prior to testing.† Four pancreatectomized dogs were studied, and none showed reduction in plasma AHF as compared to normal dogs.

Ten rats were given 0.2 cc of carbon tetrachloride by intraperitoneal injection. Twenty-four hours later they were sacrificed, and their plasma studied for blood clotting factors. The livers of these animals showed severe damage by gross and histological examination, and there was corresponding depression of prothrombin (less than 30%) and proconvertin (less than 1%). However, in conformity with the findings of Graham *et al.*(2), AHF was intact in all animals.

Summary and conclusions. Parenteral administration of ethionine markedly depressed the AHF content of the blood in rats. This effect probably represents interference with synthesis of the protein rather than increased destruction. Although other coagulation factors were affected, the ethionine action did not involve all serum proteins, as shown by paper electrophoresis. Extensive tissue damage in ethionine-treated animals was confined to the pancreas, liver and bone marrow. Neither total pancreatectomy in dogs, nor carbon tetrachloride-induced liver damage in rats had any effect on plasma AHF levels. Studies are in progress to determine a possible role of the reticulo-endothelial system.

We wish to thank Dr. Lelland J. Rather for his evaluation of pathological specimens.

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† Kindly supplied by Dr. Leslie L. Bennett and Mr. James Sutherland, University of California, Berkeley.

Method for Estimating Respiratory Surface Area of Mammalian Lungs from Their Physical Characteristics.* (21284)

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The surface area available for gas exchange in the lungs is an important factor in gas transfer between the alveoli and the blood(1,2). Present estimates of this area are based on measurements of alveolar dimensions taken from fixed histologic specimens(3-5). These estimates of total surface area are made by measuring or calculating the area/volume ratio for a small portion of lung tissue and applying this ratio to an assumed total lung volume. The volume which must be assumed is uncertain because the degree of inflation represented in the particular specimen is difficult to determine, especially if the lungs are allowed to collapse before fixation is complete. Moreover, distortion of lung architecture inevitably occurs with standard histologic methods involving dessication and embedding, and such lobular subdivisions as the air-sacs and alveolar ducts, which contribute only slightly to the respiratory surface area, have in the inflated state a much larger volume than is generally realized(6). For these reasons calculations by anatomic methods tend to result in overestimating the number of alveoli and the total lung surface.

The method of estimating lung surface described in this report is based on physical measurements of the lungs and avoids any assumption of size or configuration of respiratory structures. The calculation depends on consideration of the changes in free energy of the lungs as they are deflated. During inflation, energy is stored by deforming elastic elements, and by the creation of a large air-liquid interface. If the lungs are deflated by allowing volume equilibrium at successive pressure decrements, the stored energy released during deflation can be measured. As the volume of air in the lungs decreases from a volume V_1 to the totally collapsed volume V_0, the free energy recovered (or work done by the lungs) is given by

$$\int_{V_0}^{V_1} PdV$$

where P is the pressure relative to atmospheric. As will be shown below, it is possible to estimate the contribution of elastic energy to the total free energy and obtain the surface free energy by difference.

At equilibrium in a 2-phase system of several components, the surface area A_s is related thermodynamically to the (Helmholtz) free energy of the surface, F_s; for a plane interface (assuming a dividing layer of negligible thickness):

$$\frac{dF_s}{dA_s} = \gamma - S_s \frac{dT}{dA_s} + \sum_r \mu_r \frac{dn_r}{dA_s} \qquad (1)$$

where γ denotes the surface tension coefficient, S_s surface entropy, T ambient temperature, and the summation term includes the chemical potential μ and number of moles n for each component r. If the temperature is constant and the quantity and total composition of the associated bulk phases is independent of area, the last two terms are zero. Equation 1 may be applied to curved surfaces when the radii of curvature are very much greater than molecular dimensions(7). The radii of curvature of lung air-liquid surfaces undoubtedly exceed 10^{-4} cm and this condition is easily met. Subject to the assumptions implied above, equation 1, as applied to the lungs, may be written in its usual form $\frac{dF_s}{dA_s} = \gamma$.

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* This work supported in part by contract of the Army Chemical Corps.

† The author wishes to express indebtedness to Dr. Neville Lefcoe and Miss Martha McLaughlin, who have painstakingly obtained most of the actual experimental results from which the theory was developed; also to Dr. Jere Mead for valuable suggestions and criticisms, and to Dr. George Scatchard of the Massachusetts Institute of Technology for reviewing this manuscript.

For a determination of the surface area from experimental estimates of F_s, the relationship between γ and A_s must be known for the lungs. Because no evidence is as yet available on this point, γ will be assumed constant, which would be true for an aqueous interface. With this added assumption,

$$\int_{V_0}^{V_1} dF_s = \gamma \int_{V_0}^{V_1} dA_s$$

which states that the work done by the surface as the volume of air in the lungs decreases from V_1 to V_0 is represented by the product of the surface tension coefficient and the decrease in surface area. In our experiments V_0 is nearly zero, and the surface free energy given up by deflation of the lung from a particular volume is a measure of the total air-liquid surface which existed at that volume; that is, $As = F_s/\gamma$, where F_s and A_s refer to a particular lung volume.

The magnitude of F_s is estimated experimentally from lung pressure-volume curves in the following manner. (Details of methods and results will be published elsewhere.) Gas-free animal lungs are prepared by allowing the pulmonary blood flow to absorb the alveolar gases(8), after initially hyperventilating the lungs with pure oxygen. These completely deflated lungs are removed, filled with isotonic NaCl solution while suspended in a saline bath, and the "elastic" curve is obtained during deflation by allowing the volume to reach equilibrium at each pressure. This curve is then compared with the deflation curve subsequently obtained in the same way after air inflation at the same temperature. Typical saline and air curves for rat, cat, and dog lungs are shown in Fig. 1. The volume ordinates for the 3 species are different, but the pressure scales are the same. If we assume that the curve from the saline run gives a reliable estimate of the work done by elastic elements during deflation in air, the area between the 2 curves represents the free energy of the surface recovered as the area decreases. The surface energy is roughly ⅔ of the total for the rat, ½ for the cat, and ¼ for the dog. This species difference has been a consistent finding. The surface energy has been obtained for a specific air volume by direct measurement of the shaded areas. The particular volumes selected are approximately those the animals would have *in vivo*. The results for the three animals in Fig. 1 are: Rat, 1.23 x 10^4 ergs for air vol. of 5 cm^3; Cat, 12.4 x 10^4 ergs for air volume of 100 cm^3; Dog, 26.7 x 10^4 ergs for air vol. of 400 cm^3. The air volumes are less than the total volumes shown in Fig. 1 because of residual saline solution remaining in the lungs.

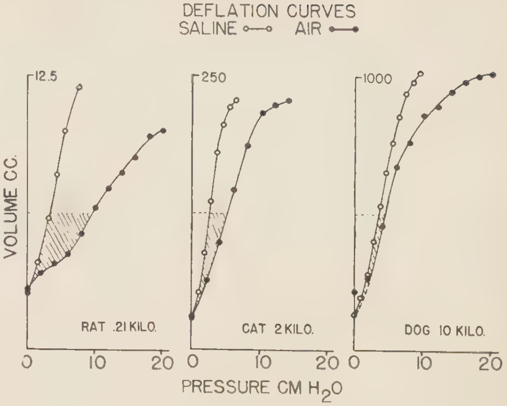

FIG. 1. Static pressure-volume curves from excised rat, cat, and dog lungs. Volumes are relative to initial volume of completely deflated lungs taken from the animal. Trapping of air due to production of a stable foam occurred in the dog experiment and lower portion of the air curve has been corrected by calculating surface energy remaining in the foam.

Aside from technical difficulties such as leaks and foaming, these values may be in error for two additional reasons. First, the "elastic" curves obtained in saline may not be strictly applicable to air-filled lungs. Although gross dimensions of the lungs are very similar during the saline and air experiments, differences in elastic strain at the alveolar level may result from the presence of an air-liquid interface. Second, theoretical considerations relating to serial closing of respiratory units during deflation(9) suggests that part of the total surface energy may be dissipated in frictional losses as closure occurs. The first source of error would result in overestimating the surface energy while the second would result in underestimating it; thus, the two tend to nullify each other. The second factor is probably the more important of the two at low levels of inflation, and the free energy estimated by integration may therefore be too small at moderate lung volume. There is no reason to believe, however, that these sources of error are very significant.

Only the surface tension coefficient remains to be considered in order to calculate the surface area. No direct measurements of γ in the lungs have been made. An attempt at estimating γ for animal lungs was made by von Neergaard(10) who measured the surface tension coefficient of lung tissue extracts, using the method of Lenard *et al.*(11). His results varied from 35 to 41 dynes/cm; in view of errors of the method(12) and the presence of intracellular substances, these values cannot be applied to intact lungs. von Neergaard found no conclusive evidence for a surface-active film in the lung, and concluded that the interfacial layer is probably an aqueous solution having a surface tension coefficient near that of serum. Human blood serum has a static surface tension coefficient of about 50 dynes/cm(13), and this value will be used for the lungs until better data are available. The figure of 50 may be too low to apply to our experiments, since the lungs have been flushed with saline ($\gamma = 72$), but colloidal solutions such as serum maintain their static surface tension even with high dilution(14).

The lung surface areas for the 3 animals of Fig. 1, as calculated from the formula $A_s=F_s/50$, are: rat, 246 cm^2; cat, 2480 cm^2; dog, 5340 cm^2. These results apply to the particular volumes at which F_s was measured.

The surface area of human lungs may also be estimated by this method. On the assumption that the pressure-volume relationship is linear and that the transmural pressure is 6 cm-of-water for a lung volume of 3500 cm^3, the total work done, F_T, during complete deflation is $F_T=\frac{1}{2}PV=\frac{1}{2} \times 6 \times 980 \times 3500=10.29 \times 10^6$ ergs. If one-fourth of this energy is surface energy (as is approximately the case for the dog of Fig. 1), then $F_s=\frac{1}{4}F_T=2.57 \times 10^6$ ergs. Assuming $\gamma=50$ dynes/cm, $A_s=F_s/\gamma=5 \times 10^4$ cm^2 or 5 m^2 at a lung volume of 3.5 liters. Since the total surface energy may be somewhat larger than estimated above (assuming an error of 100 per cent which is very liberal), the true surface area may lie between 5 and 10 m^2.

The striking difference between the estimate for human lungs of 5 to 10 m^2 by the method discussed in this report and the estimates of 50 to 100 m^2 obtained from histologic measurements, deserves additional comment. None of the assumptions on which the surface energy method depends could account for this difference unless the lung surface were a semisolid phase or consisted of a highly surface-active substance. Neither of these possibilities is at all likely. On the other hand, some of the problems in interpreting dimensions taken from fixed lung specimens have already been mentioned. For example, Willson(4) and Gertz(5) used dimensions taken from collapsed lungs in their calculations; to obtain the total area they assumed lung volumes of 3000 to 3500 cm^3. These values are much too large—a better range would be 600 to 1000 cm^3(15). If the latter figures had been used, their calculations would have yielded 7 to 20 m^2. Errors in estimates from anatomic measurements may also have arisen because in collapsed lungs the size of the air-sacs and alveolar ducts is grossly distorted, and the alveoli appear to occupy most of the lung volume. The results obtained by the method given in this report are consistent with the idea that in inflated lungs the alveoli are actually sections of spheres superimposed on large air-sacs and alveolar ducts, the volume of the alveoli representing only a small part of the total lung volume.

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Metabolism of Benzoic Acid in Normal and Irradiated Rats.*[†] (21285)

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(Introduced by J. N. Stannard.)

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In a previous communication, it has been shown that the "free glycine pool" increases in size after rats have been exposed to total body x-radiation(1). These conclusions were based on determinations, before and after irradiation, of the isotope concentration of the urinary hippuric acid excreted after injection of C-14 labeled glycine and benzoic acid. The experimental technic was modified somewhat to test the ability of irradiated rats to conjugate labeled benzoic acid. The conjugation process may be looked upon as a 2-step process involving coenzyme A (Co A) and transesterification at the S-acyl bond(2).

Benzoic acid + acetyl — Co A→Benzoyl-Co A + acetic acid(1)

Benzoyl-Co A + glycine→Benzoyl glycine + Co A(2).

Studies with benzoic acid-carboxyl-C^{14} revealed that radiation did not interfere with hippuric acid synthesis. It was noted also that a marked dilution of the specific activity of urinary hippuric acid occurred in starved animals and this suggested that there must be an endogenous source of benzoic acid. The findings to be reported in this paper indicate that benzoic acid may be the end product of an as yet little explored metabolic pathway of one of the aromatic amino acids, *e.g.*, phenylalanine.

Experimental. Four of 8 Wistar-type rats, all weighing 150-175 g, were exposed to 700 roentgens of x-rays. Immediately after the irradiation procedure, all were given an intraperitoneal injection of sodium benzoate-carboxyl-C^{14} followed by 2 more injections at 24-hour intervals. The injected solutions contained 18.3 mg of sodium benzoate. The specific activity of the material used in the initial dose was 22,100 c.p.m. per mg carbon and that used in each of the 2 succeeding doses was 18,000 c.p.m. per mg carbon. The rats were placed in confining metabolism cages and urine (without fecal contamination) was collected at daily intervals. The animals were not fed during the 3-day period of study and were not given water except for the 10 ml of saline in which each daily dose of sodium benzoate was dissolved. A description of the animals and irradiation procedure used is given elsewhere(1). The total amount and the specific C^{14} activity of the urinary hippuric acid was determined according to the method of Gaffney and coworkers(3). The C^{14}-activity of the unconjugated urinary benzoic acid was determined by measuring the radioactivity of the appropriate spot on the paper chromatograms used for the assay of hippuric acid. The total quantity of the excreted benzoic acid was not determined chemically.

Results. The data obtained are presented in Table I. Average values as well as ranges

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TABLE I. Incorporation of Benzoic Acid-Carboxyl-C^{14} into N-Benzoyl Glycine.

Collection period after isotope admin.		Urinary excretion of total C^{14} activity admin. As hippuric acid, % (1)	As benzoic acid, % (2)	Micromoles of hippuric acid excreted (3)	C^{14} activity* of hippuric acid (4)	Expected C^{14} activity of hippuric acid (5)	RIC† (6)
1st 24 hr	Irradiated	75.9	2.53	108.5	10800	17400	.49
		70.7–84.2	1.37– 3.06	104.9–111.6	10287–11073		.46–.50
	Control	64.4	6.60	104.5	9580	"	.43
		59.4–71.6	3.43–10.73	102.7–106.6	8647–10688		.39–.48
2nd 24 hr	Irradiated	58.8	8.02	105.3	7060	14000	.39
		51.5–67.2	5.92–10.74	102.1–103.8	6528– 7866		.36–.44
	Control	76.9	7.06	105.3	8930	"	.50
		62.2–85.0	4.82– 9.16	102.1–108.8	7713–10156		.43–.56
3rd 24 hr	Irradiated	61.2	7.86	97.5	8140	"	.45
		51.7–76.7	5.62–10.19	92.1–105.5	7633– 9216		.42–.51
	Control	74.6	8.67	99.9	9471	"	.53
		66.6–83.9	6.36–11.58	96.5–104.4	8653–10953		.48–.61

* C^{14}-activity = Counts/min./mg carbon.

† Relative isotope conc. = $\frac{\text{c.p.m./mg carbon of hippuric acid isolated}}{\text{c.p.m./mg carbon of benzoic acid injected}}$.

of the experimental and control animals are shown in columns 1-4 and 6. For purposes of comparison with column 4, the expected isotope concentration of the hippuric acid is given in column 5. This theoretical value was calculated on the assumption that the introduction of the 2 inert carbon atoms of the glycyl residue (*cf.* reaction 2) constitutes the only dilution of the C^{14}-activity. The fact that the labeled benzoate is not excreted quantitatively has not been taken into consideration.

Evaluation of the data in Table I shows that radiation exposure has no apparent effect on the ability of rats to conjugate benzoic acid. Although the total amount of hippuric acid excreted by the irradiated and control rats was not altered during the period of study, the isotope concentration of the hippuric acid excreted by irradiated rats is somewhat less than that of the controls of the second day.

Of more interest is the observation that there is far greater dilution of the specific C^{14}-activity of hippuric acid than could be accounted for, except by postulating a source of benzoic acid in addition to that administered. The fact that the isotope dilution does not fall during a three day period of starvation suggests that food is unlikely to be the unknown source of benzoic acid.

It is of interest to mention other observations made in relation to benzoic acid metabolism. Subsequent experiments have indicated (a) that rats on a benzoic acid-free diet continue to excrete hippuric acid in amounts comparable to that excreted when they are on a Purina fox chow diet. (b) Hippuric acid has been isolated from the urine of rats on a high aureomycin, benzoic-acid-free diet as well as (c) in urine samples excreted by rats on the second day after complete removal of the alimentary tract. It has been observed (d) that hippuric acid is present in the second urine sample excreted after birth by human babies(4).

Discussion. It has been mentioned that these studies were undertaken to ascertain whether radiation exposure impairs the ability of rats to conjugate benzoic acid. It seems clear from the data that there is no serious interference with benzoic acid conjugation during the early post-irradiation period demonstrable under these conditions of irradiation and testing. During this early period after exposure to x-rays changes in the "free-glycine" pool have been observed(1).

The observation of the dilution of the injected labeled benzoic acid by a factor of 1.5-2.0 even in the starved rats was not an entirely unexpected finding since it has been observed for some time that animals excrete hippuric acid even when benzoic acid has not been administered. Although certain forms of bacteria are known to have the ability to produce benzoic acid, this substance has not been described as a normal metabolite in vertebrates. Current isotope studies together with the observations reported in this paper provide evidence for believing that there is an endogenous source of benzoic acid which is not dependent upon food intake or on the activity of intestinal bacteria. The most likely precursor is one of the aromatic acids and, indeed, preliminary studies suggest that phenylalanine is one of the amino acids involved(5).

Summary. 1. The isotope concentration of the urinary hippuric acid of starved irradiated or non-irradiated rats given benzoic acid-carboxyl-C^{14} is much less than the dilution expected from the conjugation of the injected labeled benzoic acid with inert carbon atoms of the glycyl residue. 2. Evidence is presented that benzoic acid is a normally occurring metabolite in rats. 3. The effect of x-radiation on the ability of starved rats to conjugate benzoic acid-carboxyl-C^{14} has been tested and no defect detected under the experimental conditions used.

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Action of Poly-α-Amino Acids on Clotting of Fibrinogen by Thrombin.[*]

(21286)

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It was shown previously that basic polyamino acids inhibit blood thromboplastic activity(1), fibrinolysis(2), proteolytic activity of pepsin(3), clearing of alimentary lipemia by heparin(4), as well as bacterial(5) and viral growth(6). Acidic polyamino acids neutralized the inhibitory actions of basic polyamino acids.

In this communication it is reported that the basic polyamino acids, polylysine, polyornithine and polyarginine, accelerated the clotting of fibrinogen by thrombin, while the acidic polyamino acids, polyaspartic, polyglutamic and polycysteic acids retard the clotting.

Materials and methods. Fibrinogen. Bovine Fraction I[†] was used or purified by the procedure of Laki(7). The final preparation contained 88-93% clottable protein and was electrophoretically homogenous. Mobility calculated from the descending boundary pattern was 1.8×10^{-5} cm^2 V^{-1} $sec.^{-1}$ at pH 7.4, ionic strength 0.15 and $t = 4°C$. Determinations of fibrinogen were made by the procedure of Ware *et al.*(8).

Thrombin. A thrombin solution was obtained by adding 19 ml of isotonic buffer solution (see below) and 1 ml of 0.1 M sodium oxalate to a 1000 unit vial of Bovine Thrombin (Upjohn). One g of $BaSO_4$ (Merck) was added, the suspension was stirred for 15 minutes, centrifuged and the sediment discarded. This treatment reduced the protein content of the thrombin solution by approximately 20% without affecting the total thrombin activity. The solutions obtained showed an activity of approximately one unit of thrombin per 0.04 mg protein. Electrophoretic examination of the untreated thrombin solution carried out at pH 7.4, ionic strength 0.15 and t = 4°C revealed 4 components with mobilities similar to those found by Seegers *et al.*(9). After treatment with $BaSO_4$ the fast moving component disappeared from the electrophoretic pattern.

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[†] Obtained through the courtesy of Mr. Lawrence L. Lachat of Armour & Co., Chicago, Ill.

Isotonic buffer solution. 10 ml of 0.1 M sodium veronal-HCl buffer (Michaelis) pH 7.4 were added to 90 ml of 0.9% NaCl solution. The ionic strength of the final buffer solution was 0.15. All substances were dissolved in and diluted with this medium.

Polyamino acids were obtained from the Department of Biophysics of the Weizmann Institute of Science. They included (n indicates average chain length): Poly-DL-lysine hydrobromide n=36(10), poly-DL-ornithine hydrochloride n=29(11), poly-DL-arginine sulphate n=20(11), poly-L-aspartic acid n=150(12), poly-glutamic acid n=49(13), poly-L-cysteic acid n=10(14) and poly-DL-alanine n=30(15). The basic polyamino acids were dissolved in the isotonic buffer solution. The acidic polyamino acids were first dissolved in 0.15 N NaOH, the solution was neutralized with 0.15 N HCl and 1/9 volume of 0.1 M veronal-HCl buffer pH 7.4 was added. The concentrations of the basic polyamino acids given in μg/ml refer to the concentration of the respective polycationic salts.

Protamine sulfate from Nutritional Biochemicals Corp. was used.

Heparin (Upjohn) contained chlorobutanol as a preservative. Control tests revealed that this substance did not affect clotting of fibrinogen.

Platelet-free plasma. Human blood was collected by an all-silicone technique, mixed with 1/19 volume of 0.2 M sodium oxalate, centrifuged at 18,000 g for 15 minutes at 4°C and the supernatant separated.

Clotting times were measured at 37°C in glass tubes of 11 mm internal diameter. The first appearance of macroscopically visible fibrin strands was taken as the end point. Upon addition of basic polyamino acids and protamine to fibrinogen the solutions became turbid but this did not interfere with the reading of the end point.

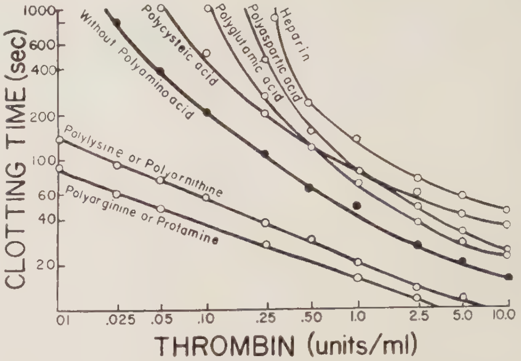

FIG. 1. Effect of 200 μg/ml of various poly-α-amino acids and of heparin on time of clotting of Fraction I by thrombin solutions of different strengths.

Clotting mixtures, when not stated otherwise, consisted of 0.2 ml of fibrinogen solution, 0.2 ml of solution of test substance or isotonic buffer solutions and 0.2 ml of thrombin solution which was added last. All concentrations are given per ml of final clotting mixture.

Experimental. The accelerating effect of the basic polyamino acids and protamine and the retarding effect of the acidic polyamino acids and heparin on the time of clotting of Fraction I by different strengths of thrombin is shown in Fig. 1. Similar concentrations of the neutral polyamino acid polyalanine and of the monomers of the respective polyamino

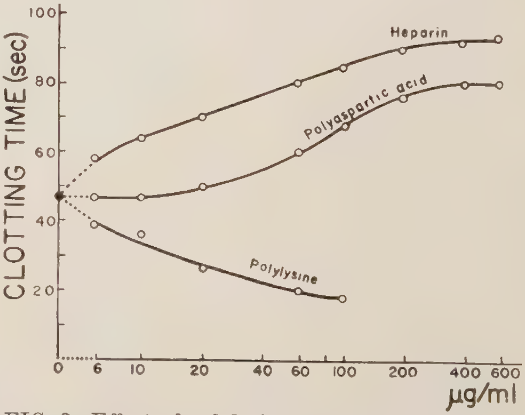

FIG. 2. Effect of polylysine, polyaspartic acid and of heparin in various concentrations on time of clotting of purified fibrinogen (2.5 mg/ml) by thrombin (1 unit/ml).

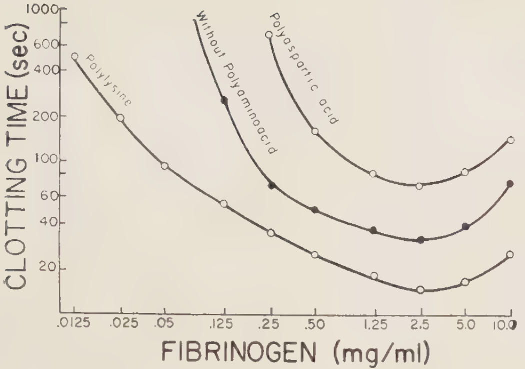

FIG. 3. Effect of 100 μg/ml of polylysine and polyaspartic acid on time of clotting of varying concentrations of purified fibrinogen by 1 unit/ml of thrombin.

acids as well as the basic dyes toluidine blue and diazine green did not influence the clotting time. The clot accelerating action of polylysine and the clot retarding action of polyaspartic acid and heparin increase with the rise in concentration (Fig. 2).

The effect on clotting time of varying fibrinogen concentration in the presence of a constant concentration of polylysine and polyaspartic acid respectively is shown in Fig. 3. Low concentrations of fibrinogen which were not clotted by thrombin after several hours were clotted within minutes in the presence of polylysine. This may be useful for the detection of very low concentrations of fibrinogen.

It is known that heparin combines with a plasma co-factor to give a complex with marked antithrombin activity. If the fibrinogen preparation used contained such a co-factor, an antithrombin complex between the co-factor and heparin would be formed (a similar complex might possibly be formed also in the case of polyaspartic acid). The clotting times in the presence of this complex would be significantly prolonged. The following experiment was designed to investigate this possibility.

Two series of experiments were performed in which either platelet-free plasma or purified fibrinogen were mixed with polylysine, polyaspartic acid or heparin. In each case thrombin was added either immediately or after preincubation of the mixture at 37°C for a period of 10 minutes, and clotting time was measured (Table I). Polylysine accelerated clotting of both plasma and purified fibrinogen solution. Polyaspartic acid retarded clotting of purified fibrinogen but had no significant effect on the plasma clotting times. In the case of polylysine and polyaspartic acid preincubation with purified fibrinogen as well as with plasma had no effect on clotting times. In the case of heparin however it can be seen that preincubation increased the clotting times of platelet-free plasma considerably, while it had no effect when purified fibrinogen was used. (Table I). It should be noted that at the

TABLE I. Effect of Preincubation of Platelet-Free Plasma or Purified Fibrinogen with Polylysine, Polyaspartic Acid or Heparin on Time of Clotting by Thrombin.

		Clotting times (sec)			
		Platelet-free plasma*		Purified fibrinogen*	
Test substance	Final conc., μg/ml	Without preincubation	After preincubation	Without preincubation	After preincubation
Isotonic buffer control		11	10	16	16
Polylysine	100	6	6	11	11
Polyaspartic acid	400	10	9	24	25
Heparin	.5	13	15	16	16
	1	26	30	17.5	17
	2	34	42	21	20
	3	77	95	23	22
	5	180	420	24	24
	7.5	380	960	25	26
	15	900	∞	27	28
	30	∞	∞	27	28

* Containing 4.5 mg fibrinogen/ml.

To .2 ml substrate .1 ml test substance was added. After 10 min. incubation at 37°C .2 ml of 9 units/ml thrombin together with another .1 ml test substance were added. The latter was added in order to keep its concentration constant throughout the experiment.

TABLE II. Neutralization of the Clot Accelerating Action of Polylysine by Polyaspartic Acid.

Polyamino acids in clotting mixture*	Clotting time (sec)
Control (isotonic buffer without polyamino acid)	42
Polylysine	21
Polyaspartic acid	60
Polylysine + polyaspartic acid premixed	43
Polyaspartic acid added to polylysine—fibrinogen mixture	41
Polylysine added to polyaspartic acid—fibrinogen mixture	43

* Containing .2 ml purified fibrinogen (7.5 mg/ml), .2 ml thrombin (3 units/ml) and .1 ml of polyamino acid solutions indicated in the table (polylysine 600 μg/ml, polyaspartic acid 1200 μg/ml); the mixtures were made up to a final volume of .6 ml.

same heparin concentration plasma clotting times were markedly longer than with purified fibrinogen.

The antithrombin activity of plasma in the presence of heparin is known to increase markedly during the first few minutes of incubation(16,17). As no such increase was noted in the case of the purified fibrinogen used it may be concluded that it is devoid of the plasma heparin co-factor. The clot retardation by polyaspartic acid and heparin seems therefore to be the result of a direct action on the conversion of fibrinogen to fibrin by thrombin. This is consistent with the observations of Klein and Seegers(18) on the clot retarding action of heparin.

The clot accelerating effect of polylysine was abolished by addition of polyaspartic acid, and the retarding effect of polyaspartic acid was abolished by addition of polylysine (Table II). This neutralization also took place even after the reaction was allowed to proceed (Table III).

Discussion. The clotting of fibrinogen by thrombin and by coagulase-thrombin(19) is the first enzymatic reaction found to be accelerated by the basic synthetic polyamino acids, already known to be inhibitors of a variety of biological systems. In preliminary experiments it was observed that the clotting of fibrinogen by papain is also accelerated by basic polyamino acids(20). Toluidine blue and diazine green, basic dyes with low molecular weight, which—like basic polyamino acids—neutralize heparin and inhibit thromboplastic activity, did not exhibit any activity in the above reaction. It appears therefore that the polycationic nature of the polymers employed plays an important role in their accelerating action. This is also apparent from the fact that the natural basic polypeptide, protamine, exhibits "fibrinoplastic" activity(21).

The clotting of fibrinogen by thrombin involves splitting off from fibrinogen of an acidic polypeptide rich in aspartic and glutamic acid residues(22). The thread-like basic polylysine molecules may combine with the acidic polypeptide either when released or while still attached to the fibrinogen molecule. Such a combination may reduce the repulsive forces between negatively charged thrombin and fibrinogen and facilitate the approach of the enzyme to the substrate. The possibility that basic polyamino acids combine with thrombin, or both thrombin and fibrinogen, should also be considered.

The neutralization of the clot accelerating action of the basic polyamino acid polylysine by the addition of acidic polyaspartic acid to the clotting mixture is probably the result of the formation of a biologically inactive complex due to combination between the oppositely charged polyamino acids.

Summary. 1. The synthetic basic poly-α-

TABLE III. Neutralization of the Clot Accelerating Action of Polylysine by Polyaspartic Acid Added at Various Time Intervals to the Fibrinogen-Polylysine-Thrombin Mixture.

Time* interval after which polyaspartic acid or buffer was added	Clotting times* of mixtures†			
	Containing polylysine +		Without polylysine +	
	Poly-aspartic acid	Buffer sol.	Poly-aspartic acid	Buffer sol.
0	42	22	58	40
5	41.6	21.8		
10	41.0	21.4		
15	39.5	21.0		
20	37.8	20.8		

* Seconds after addition of thrombin.

† Containing (in order of addition):
.2 ml purified fibrinogen sol. 7.5 mg/ml,
.1 " polylysine 600 μg/ml or .1 ml buffer sol.,
.2 " thrombin sol. 3 units/ml,
.1 " polyaspartic acid 1200 μg/ml or .1 ml buffer sol. (added at various time intervals).

amino acids, polylysine, polyornithine and polyarginine, accelerate conversion of fibrinogen to fibrin by thrombin; the acidic poly-α-amino acids, polyaspartic, polyglutamic and polycysteic acids, and also heparin, retard this reaction. 2. The acidic polyamino acids neutralize the clot accelerating activity of the basic polyamino acids. 3. The addition of polylysine makes possible the detection of concentrations of fibrinogen too low to be clotted by thrombin alone.

We should like to express our indebtedness to Mr. M. Breuer for help in electrophoresis experiments, and to Miss A. Herzog for her valuable assistance.

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Inhibition of Degradation of Insulin-I^{131} *in vivo.** (21287)

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The processes of degradation and of inactivation of insulin by tissues *in vitro* can be inhibited by a number of substances(1,2). Both *in vitro* and *in vivo* the system in which degradation is accomplished does not distinguish between insulin and I^{131}-labeled insulin(1,3). It would seem to be of great importance to determine if the degradation of insulin-I^{131} can be inhibited *in vivo,* because such inhibition might effect potentiation of insulin action and have therapeutic implications. Accordingly, the effects of the following compounds on the degradation of insulin-I^{131} *in vivo* were studied.

(1) *Zinc.* There has been a great deal of study of the chemical and biological interaction between insulin and zinc, and in practice, zinc has been used as a factor in various insulin preparations. It is of interest then, that zinc has been shown to be a potent inhibitor of insulin degradation(1) and inactivation(2) *in vitro,* and accordingly the

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† U. S. Public Health Service Research Fellow.

effect of zinc on the *in vivo* degradation of insulin-I^{131} was studied.

(2) *Polyphloretin phosphate.* The enhancement of the ascorbic acid depleting activity of adrenocorticotrophin by the systemic administration of polyphloretin phosphate, (PPP), has been ascribed to anti-enzymatic properties of this substance(4). In view of (a) possible similarities between insulin and adrenocorticotrophin metabolism(5) and (b) the evidence that insulin-I^{131} degradation is proteolytic(1), we desired to find out if PPP affected this degradation.

(3) The effect of *5-isoproplyidene-2,4-dithiohydantoin* (MC 2346) on insulin-I^{131} degradation was investigated because of the report by Houssay *et al.*(6), that this compound reduced the incidence of diabetes in subtotally pancreatectomized rats.

Methods. The method used to measure the degradation of insulin-I^{131} was based on the following observations. The radioactivity of insulin-I^{131} is protein-bound‡, but after its injection into a rat, this property is rapidly lost (5). A fraction of radioactivity appears that is not protein-bound as measured by trichloracetic acid(TCA) precipitation, and thereby represents degradation of the insulin-I^{131}. A standard procedure, described elsewhere(5), has been adopted to measure insulin degradation and was used in these experiments. A brief outline of the method follows. Sprague-Dawley male rats of similar weight and age were used in all studies. Seventeen hours before an experiment, experimental and control animals had their food and water replaced by 10% dextrose in 0.9% saline (10% D/S). This was removed and the animals fasted for 2 hours before the experiment was begun. Insulin-I^{131} was then injected intravenously in all rats of both groups, and 15 minutes later, the animals were sacrificed and gastrocnemius muscle, blood, liver, and kidney removed. The radioactivity of these tissues was divided into a protein-bound fraction and a supernatant fraction by means of TCA precipitation. Concentration of radioactivity in each fraction was expressed as [T]/[B], a ratio of the concentration of radioactivity in the fraction to the initial overall total body concentration. Values greater than one therefore represented concentrations greater than the initial total body concentration, and values less than one represented lesser concentrations.

Results. Zinc was given to rats in 3 separate experiments. In one experiment, zinc acetate, 0.01 g in 0.2 ml of distilled water, was given to each of 5 rats and sodium acetate, 0.008 g in 0.2 ml distilled water was given to each of 4 control rats. These doses were given subcutaneously twice, once 17 hours before, and finally one hour before the injection of 0.5 unit, 10 μc of insulin-I^{131}. A second experiment was done in 2 parts. Both involved the feeding of zinc carbonate in the diet. A 0.5% Zn diet (Purina Laboratory Chow containing 0.5% zinc as zinc carbonate) was given to 4 rats for 2 weeks and to 5 rats for 4 weeks. Equal numbers of controls of the same weight and age received chow without added zinc for the same intervals. At the termination of the experiments all were given 0.3 unit (10 μc) of insulin-I^{131}. In all of these experiments there was no significant effect of the administration of zinc on the distribution or degradation of insulin-I^{131}.

Polyphloretin phosphate.§ Two experiments were performed using this substance. In one, 8 rats were given 2 mg of PPP per kg intramuscularly in a 1% solution in 0.9% saline 30 minutes before insulin-I^{131} injection and compared with 7 controls who received 0.5% saline only, before the insulin-I^{131}. In the second experiment 40 mg of PPP per kg in a 2% solution in 0.9% saline was given intramuscularly 17 hours and one hour before insulin-I^{131} injection. Again controls of the same age and weight received 0.9% saline only. In both experiments there was no effect of PPP on the distribution or degradation of insulin-I^{131}.

5-isopropylidene-2,4-dithiohydantoin.||

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‡ The insulin was kindly provided by Eli Lilly and Co., and iodinated in the Abbott Laboratories at Oak Ridge. In the preparation of the material, unbound I^{131} was removed by dialysis. Essentially all of the radioactivity remaining was then precipitable with trichloracetic acid.

§ The PPP was kindly supplied by Dr. Gustav Martin of the National Drug Co.

|| The MC 2346 was kindly supplied by Mr. W. A. Lott of E. R. Squibb and Sons.

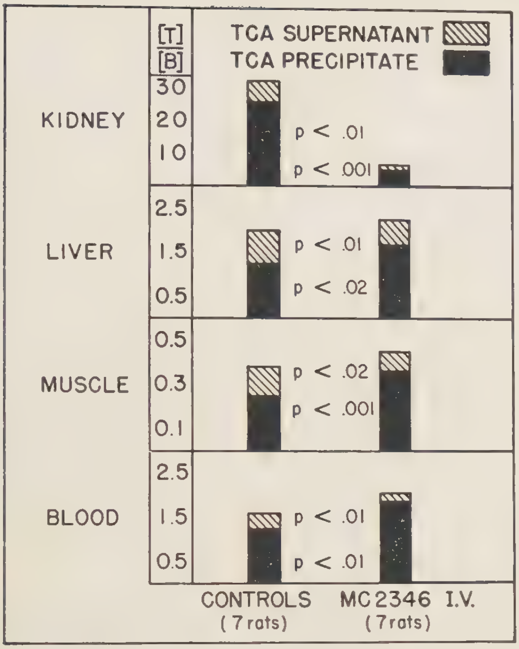

FIG. 1. Effect of 5-isopropylidene-2,4-dithiohydantoin (MC 2346) given intravenously, on concentrations, $\frac{[T]}{[B]}$, of radioactivity in trichloracetic acid (TCA) precipitate and supernatant fractions of tissues 15 min. after intravenous administration of insulin-I^{131} to rats. Figures between columns are p values of differences between respective fractions of treated and control groups.

Three experiments were performed with this substance: 1) Seven rats weighing between 166 and 198 g were each given one ml of a 2% suspension of MC 2346 in 0.9% saline alkalinized to pH 8.9 (alkaline saline) intravenously 2 minutes before the injection of one unit, 10 μc of insulin-I^{131}. Seven controls, weighing between 146 and 192 g, received one ml of the alkaline saline alone and similarly, 2 minutes later, insulin-I^{131}. The results are shown in Fig. 1. Profound changes resulted from the injection of the MC 2346. Supernatant radioactivity was reduced in all tissues. Bound radioactivity was increased in liver, muscle, and blood, and decreased in kidney. We interpret these observations as suggestive that MC 2346 decreased insulin-I^{131} degradation.

2) Six rats weighing between 132 and 174 g were each given one dose of 25 mg of MC 2346 in one ml of alkaline saline by stomach tube 4 hours before insulin-I^{131} injection. Five controls weighing between 155 and 172 g were given alkaline saline only, by stomach tube, and 4 hours later were injected with insulin-I^{131}. The results of this experiment showed no effect of the single oral dose of MC 2346 on the distribution or degradation of insulin-I^{131}.

3) Five rats, weighing between 135 and 171 g were given daily for 5 days the same dose of MC 2346 as described in the previous paragraph, with the last dose being given 4 hours before the injection of the insulin-I^{131}. Five controls, weighing between 152 and 206 g received only alkaline saline solvent in the same dosage schedule. Both groups received one unit, 10 μc, of insulin-I^{131} intravenously. The results are shown in Fig. 2. The changes appear to be similar to those following the administration of MC 2346 intravenously, although perhaps not as marked.

Discussion. The tolerance of the rat for

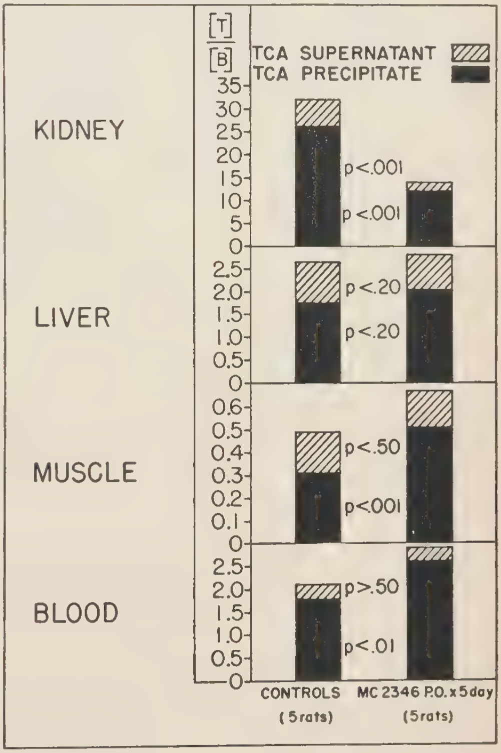

FIG. 2. Effect of prolonged oral administration of MC 2346 on concentrations, $\frac{[T]}{[B]}$, of radioactivity in tissue fractions 15 min. after intravenous insulin-I^{131} in rats.

zinc has been considered to be between 0.5 and 1% of the diet by weight(7). At the lower level, reproduction was found to be impaired, but growth was not. Accordingly this level seemed to be a suitable one for the study of the effect of zinc on insulin-I^{131} metabolism *in vivo*. It was found, however, to be without effect, even after 4 weeks of such a diet. This may mean that different conditions *in vivo* counteract or prevent the inhibitory effect of zinc which has been seen *in vitro*, or perhaps that different dosage programs need to be investigated.

The dose levels of PPP used were ones that have been shown to potentiate adrenocorticotrophin action. The absence of an effect of this dosage on insulin-I^{131} degradation is then of significance and raises the question of the mechanism of action of PPP with respect to adrenocorticotrophin.

The action of MC 2346 on insulin-I^{131} degradation is of great interest, particularly since this substance has been shown to prevent, at least partially, the development of diabetes(6) in the subtotally pancreatectomized rat. The present study has indicated that MC 2346 decreases the degradation of insulin. It seems possible that these 2 functions are related, and, as has been suggested before(5), that decreased insulin degradation results in decreased insulin inactivation. Less insulin can exert the same amount of action as is exerted by a larger quantity which is in the presence of a fully functionary degradation system. The insulin insufficiency resulting from subtotal pancreatic resection may be alleviated by MC 2346 because of a reduction in insulin degradation. Such a concept offers promise of a new therapeutic approach to the insulin insufficiency of diabetes mellitus. Perhaps an inhibitor of insulin degradation could so bolster a relative insulin insufficiency as to make endogenous insulin adequate, and restore physiologic homeostasis.

Summary. Insulin-I^{131} degradation in rats *in vivo* was not inhibited by the administration of zinc in the diet, or by treatment with the substance, polyphloretin phosphate. Marked inhibition was obtained by the intravenous or prolonged oral administration of 5-isopropylidene-2,4-dithiohydantoin and it was suggested that this may explain the protection afforded against diabetes by this substance in the partially depancreatized rat.

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Polyvinylpyrrolidone (PVP) as Adjunct in Centrifugal Separation and Enzymic Assay of Subcellular Components.* (21288)

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Sucrose solutions have long been used in the centrifugal separation of subcellular components, the sucrose concentration varying with the tissue and ends sought(1-4). Sucrose tends to maintain morphologic and enzymic integrity of subcellular particulates. However, in certain tissues separation of larger particulates (nuclei, mitochondria) from the microsomal fraction is made difficult by extensive agglutination of the latter. This may occur even in the absence of added electrolytes. In such cases it is often difficult, or impossible, to remove microsomal contamination from the mitochondrial fraction even by repeated resuspensions in fresh medium. This difficulty, which was encountered in mouse brain and tumors (melanoma, hepatoma) was materially overcome by adding a polymer of 1-vinyl-2-pyrrolidone (polyvinylpyrrolidone or PVP) to the sucrose solution.[†] PVP, presumably in part by increasing colloid osmotic pressure of the medium, counteracts irreversible agglutination of microsomes, facilitates centrifugal resolution of larger cell components (mitochondria, nuclei, etc.) and acts as a metabolic stabilizer. This paper briefly reports a method of using PVP which permits relatively simple separations of subcellular components of mouse brain, liver, and tumor (melanoma, hepatoma). Many modifications in procedure are feasible, but their desirability will depend upon the particular tissue and problems involved. PVP aids in preserving mitochondrial form, and has been used to advantage in studies of tissue slice metabolism(5).

Experimental. Freshly prepared 10% solutions of PVP in 20% sucrose (g/100 ml of aqueous solution) had a pH of about 4. These were adjusted to pH 8-8.5 with KOH. Fragments of mouse brain macerated in this medium, and examined by phase contrast microscopy, showed clean well preserved mitochondria and nuclei with little aggregation and no evidence of microsomal agglutination. On the other hand, brain macerates prepared in 10-30% sucrose solution developed extensive microsomal agglutination (evidenced by formation of a fine gray precipitate visible with phase contrast microscopy). Those prepared with PVP alone, without sucrose, showed swelling of mitochondria (5 to 10% PVP) or poorly dispersed cellular components (20% PVP).

Homogenates. Enzymic activities of brain

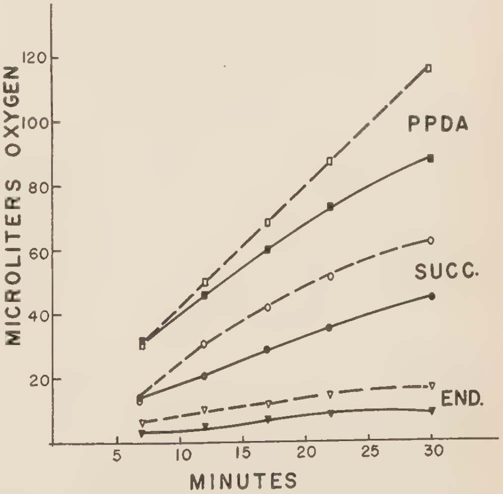

FIG. 1. Cumulative curves for microliters of oxygen consumed/0.6 ml of mouse brain homogenate in 20% sucrose (solid lines), or in 20% sucrose plus 10% PVP (broken lines). PPDA, 10% homogenate with 0.05M paraphenylene diamine plus 2.4×10^{-4} M cytochrome C. SUCC., 10% homogenate with 0.05M succinate plus cytochrome C. END., 20% homogenate with cytochrome C but no added substrate. (100% O_2, plus 2N KOH, in center wells, 38°C)

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[*] This study reports work carried out in both the National Microbiological and National Cancer Institutes of the National Institutes of Health.

[†] The possible use of polyvinylpyrrolidone for subcellular extractions was suggested to the author in 1951 by Dr. Harold A. Levine. Special thanks are due to Mr. Daniel B. Witwer of the General Aniline and Film Corp., Easton, Pa., for making available at that time an imported sample (1687-202) of PVP and later samples of domestic manufacture (mol. wt. ca. 40,000). All were satisfactory.

homogenates simultaneously prepared in 20% sucrose, or in 20% sucrose plus 10% PVP, are illustrated in Fig. 1. These, and other measurements, showed that PVP had no deleterious effects on enzyme activity, provided, in the case of aerobic systems, an adequate oxygen tension was maintained. In some instances respiration in PVP was actually higher than in plain sucrose (Fig. 1).

Centrifugal separation. The results of an experiment involving a simplified method for mouse brain will be described. Brains of 15 mice were collected in 20 ml of ice-cold 20% sucrose plus 10% PVP (pH 8). The tissue (8 ml in volume) after chilling was removed, quickly drained, and ground at 0°C in a glass Potter-Elvehjem homogenizer with 28 ml of fresh sucrose-PVP solution, and filtered with suction through glass wool. The filtrate (28.5 ml), following removal of an aliquot for enzymatic activity measurements (homog., Table I), was then fractionated at 0°C in an International refrigerated centrifuge (PR-1) with multispeed attachment.

Step (a). Two one-minute cycles were run at maximum speed (R.C.F. ca. 25000 x g). The pellets contained almost all of the red cells and nuclei together with some large mitochondria (P_1, Table I).

Step (b). Two pellets were then obtained from the cleared homogenate by centrifuging 2 aliquots for 45 minutes at maximum speed. One pellet was suspended in 10% sucrose plus 5% PVP and used for enzyme assay (P_2 unwashed, Table I). Microscopically this suspension showed well-preserved, non-agglutinated mitochondria and no nuclei. The supernatant of this run contained a few mitochondria and much microsomal material. The visible particulates together with some microsomal material were removed by a second spin at maximum speed for 40 minutes (cleared supernatant = SN, Table I).

Step (c). One of the original pellets from step b (derived from ⅔ of the cleared homogenate) was resuspended in 7 ml of 20% sucrose plus 10% PVP, rehomogenized briefly and then 7 ml of distilled water (0°C) rapidly stirred into the suspension. Following a rehomogenization the suspension was divided into 2 parts and spun at maximum velocity for 40 minutes. The supernatant contained only a trace of mitochondria, but considerable vague gray, rapidly moving material below the limit of optical resolution (resuspended microsomes). This supernatant was largely cleared of visible mitochondria by a second spin at the same speed for 40 minutes (this cleared supernatant = P_2SN_1, Table I). One of the 2 original pellets was resuspended in 10% sucrose plus 5% PVP (P_2 washed once, Table I). Microscopic analysis showed well-preserved mitochondria with little evidence of precipitated microsomal material.

TABLE I. Enzymic Activities of Subcellular Fractions of Mouse Brain Isolated in Sucrose-PVP and Expressed as Microliters Gas Exchange/Hour/ml of Homogenate Equivalent (12.5% Tissue by Vol.).

	Cytochrome oxidase activity*		Anaerobic glycolytic activity†	
Fraction	µl O_2	% of homog.	µl CO_2‡	% of P_2, unwashed
Homogenate	327	100	—	—
P_1	70	21	—	—
P_2, unwashed	364	111	801	100
P_2, washed once	314	96	533	67
” ” twice	322	98	568	71
P_3	17	5	63	8
P_2SN_1	0.5	0.1	62	8
P_2SN_2	0	0	27	3
SN	8	2	35	—

* Vessels contained 0.03M PPDA, 4.8×10^{-4}M cytochrome C., 100% O_2 + KOH, 38°C.

† Vessels contained ATP 0.0019M, DPN 0.0006 M, Mg salt HDP 0.0068M, niacinamide 0.04M, dextrose 0.04M, KH_2PO_4 0.003M, K_2HPO_4 0.008M, $MgCl_2$ 0.01M, glutathione 0.01M, and $KHCO_3$ 0.04 M (95% N_2 + 5% CO_2, 38°C).

‡ (Activity of fraction plus SN)−(activity of SN run alone) = activity of fraction in presence of supernatant (*cf.* ref. 5, p. 348).

Step (d). The other original pellet from step c was homogenized in 4 ml of 20% sucrose plus 10% PVP, 4 ml of cold distilled water added with stirring, rehomogenized, and then spun at maximum speed for 40 minutes. The supernatant (P_2SN_2, Table I) contained very few (3 or 4 per oil-immersion field) visible granules and much less microsomal material than the previous wash supernatant. The resuspended pellet (in 10% sucrose and 5% PVP) consisted almost entirely of well-preserved mitochondria (P_2 washed twice, Table I).

Step (e). The small, chiefly microsomal, clearance pellets from the supernatants of steps b and c were combined and suspended in 10% sucrose plus 5% PVP (P_3, Table I). The centrifugal procedure employed here yields a mitochondrial fraction (P_2 washed twice) nearly free of microsomes (microsomal contamination is quickly evidenced by precipitation following cautious acidification of the suspension under phase microscopy), small pellets of nearly pure microsomal material (P_3), but leaves the mitochondrial wash supernatants ($P_2SN_{1\ \text{and}\ 2}$), and the original supernatant (SN), heavily contaminated with microsomes. The microsomes can be sedimented in sucrose-PVP by centrifugation at a R.C.F. of ca. 100000 x g (Spinco centrifuge) or by addition of $MgCl_2 \cdot 6H_2O$ (1 to 2 mg per ml) and spinning at ca. 25000 x g. This latter treatment promotes agglutination of microsomes without precipitating solubilized enzymes as *e.g.*, those of the glycolytic system.

The distribution of cytochrome oxidase activities in the various fractions as determined by Warburg manometry (Table I) were as expected from the microscopic determinations. The removal of microsomal material during washing of the mitochondrial pellet (P_2 unwashed) was evidenced microscopically by inducing microsomal precipitation in the wash supernatants ($P_2SN_{1\ \text{and}\ 2}$), and by determining the glycolytic activity of these fractions in the presence of the original supernatant(5). It may be of interest to note that rabies virus in subcellular fractions obtained by sucrose-PVP fractionation of infected mouse brains was not measurably reduced in infectivity by the presence of PVP(6). A modification of the methods reported here has been used to study intracellular distribution of catalase(7).

Summary. Addition of polyvinylpyrrolidone (PVP) to sucrose solutions used in extraction of subcellular components improved centrifugal resolution of particulates, helped to preserve morphologic integrity of subcellular elements, and aided in preservation of enzymic activities. PVP was particularly useful in counteracting irreversible agglutination of microsomes.

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W - V Variation Induced in *S. typhi, S. paratyphi C* and *Paracolobactrum ballerup.* (21289)

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The Vi antigen occurs in a number of Salmonellae and other Enterobacteriaceae. Colonies with this antigen are designated as V colonies, morphologically and serologically different from W forms which lack the Vi antigen and react only with 0 sera. A change of V cultures to W, called V-W variation, is commonly observed in the laboratory. Little is known, however, about the reverse process, *i.e.*, W-V variation. It does not occur spontaneously. Booy and Wolff(1) succeeded in inducing Vi antigen in *S. typhi* W by growing it in media containing live or killed *S. typhi* V (vi) and *Ballerup* Vi organisms. Working with *Escherichia coli*, an organism related to Salmonellae, Boivin *et al.*(2) showed that transformation experiments with this microbe are successful only when unstable but smooth strains are used. This group of workers believed that the active principle involved herein is a nucleic acid or a complex containing it (3). Braun and Whallon(4) showed that in the presence of whole organisms and cell-extracts, especially of heterologous desoxyribosenucleic acid, mutants of Brucella and *E. coli* develop that ordinarily do not establish themselves.

These observations warranted two questions. First, if only *S. typhi* and *Ballerup* organisms containing a closely related Vi antigen can give impetus to the recovery of V forms from W cultures or if this can be accomplished also by adding *S. paratyphi C* with Vi antigen. Secondly, what is the role of the different fractions of Vi antigenic material in the induced W-V variation. Since desoxyribosenucleic acid (DNA) and ribosenucleic acid (RNA) seem to be of paramount importance for Enterobacteriaceae, experiments with these materials were also indicated.

Materials. Stock cultures of V strains, *S. typhi Bhatnagar I* Vi, *S. typhi* 2 *Vi, S. paratyphi C* Vi and *Paracolobactrum Ballerup* 1 Vi were maintained on egg slants. Subcultures on agar plates were made biweekly, and at least 1000 colonies checked from each transplant for V and W characteristics. Stock cultures of W strains, *S. typhi* 158 O, *S. typhi* 212 O, *S. typhi* H 901, *S. paratyphi* C O and *Paracolobactrum Ballerup* 1 O, as well as rough strains, *S. typhi* 58 R and *S. paratyphi C* R, *were* maintained on nutrient agar slants and checked for colony characteristics biweekly. For the experiments proper, stock cultures of known purity were inoculated into Tryptose (Difco, Inc.) broth, containing 1% of this peptone, and 0.5% sodium chloride, pH 7.2 Amounts of 10^{-2} ml were streaked from this medium, 20 to 22 hours after inoculation, to plates made of 1% Tryptose, 0.5% sodium chloride and 1.5% agar, pH 7.2, and the colonies observed morphologically as well as tested serologically with the aid of the slide agglutination method. Only those giving at least a 3+ reaction with Vi sera and negative with O sera were considered V colonies. Preliminary experiments showed that the most favorable incubation time for the plates was 18 to 20 hours. Heat-killed organisms, acetone precipitates, acid hydrolysates, DNA and RNA extracts were added to the Tryptose broth tubes. They were prepared according to the following procedures: The strains were grown in Tryptose broth for 20 to 22 hours, then centrifuged and the supernatant discarded. Heat-killed bacteria were prepared by suspending the organisms in 0.9 per cent sodium chloride to make a concentration of 10^7 microbes per ml saline, and keeping this suspension at 56°C in the water bath for one hour. Acetone-precipitates and saline extracts therefrom were produced according to the method of Webster *et al.*(5). The same outline was followed in making acetic acid hydrolysates. The starting material was growth in Tryptose broth collected by centrifuging. DNA and RNA extracts of the organisms were prepared by the procedure of Morse and Carter(6). Heat-killed organisms and bacterial extracts were used as soon as possible after preparation. They were added to the tubes of Tryptose broth just prior to inoculation, in concentrations equivalent to 10^6 organisms per ml of the medium. After culturing the respective strain in Tryptose broth containing heat-killed organisms or an extract for 20 to 22 hours, 5×10^{-3}% of the growth was transferred to a Tryptose broth tube with the same heat-killed organisms or with the same extract. This procedure was repeated for 14 days, streaking check-plates before each transfer.

Results. Neither heat-killed organisms nor extracts from V, W and R cultures lead to the appearance of Vi antigen in rough strains *S. typhi* 58 R, *S. paratyphi* C, R). None of the W strains formed V colonies when cultured without the addition of heat-killed organisms or their extracts in 14 successive transfers.

The relationship of V to W colonies varied but remained equal to or higher than 910 to 90 in all experiments in which heat-killed V, W or R strains were added to V strains.

None of the W strains formed V colonies when W or R cultures or extracts were added to the medium.

Acetic acid hydrolysates of V organisms were ineffective.

Heat-killed V organisms, their acetone precipitates and DNA extracts, as well as in some cases also their RNA extracts, induced the ap-

TABLE I. Induced W–V Variation : No. of Subculture in Which V Colonies Were First Demonstrated.

W strain	W organism grown in presence of:															
	S. typhi Bhatnagar I				*S. typhi* 2 Vi				*S. paratyphi* C Vi				*Paracolobactrum Ballerup* 1 Vi			
	W	A	D	R	W	A	D	R	W	A	D	R	W	A	D	R
S. typhi 158 O	2	4	4	7	3	1	2	0*	6	9	6	0	5	8	8	0
" 212 O	1	5	2	0	5	10	4	0	7	8	7	0	3	8	9	0
" 901 H	3	7	3	0	2	10	4	0	5	9	6	0	4	10	8	0
S. paratyphi C O	3	7	5	8	6	9	8	0	3	6	3	9	6	7	4	10
Pa. Ballerup 1 O	5	10	7	0	9	0	8	0	6	10	9	0	3	5	5	0

* V colonies not observed in 14 serial transplants.
W, whole organism, heat-killed; A, saline extract of acetone precipitate; D, DNA; R, RNA.

pearance of V colonies on plates streaked from Tryptose broth to which these materials were added, usually after several transfers. Table I shows the number of transfers in which this W-V change occurred.

Discussion. It was shown that W-V variation can be induced in susceptible Enterobacteriaceae by growing their W cultures in serial transfers in media containing heat-killed V organisms or certain extracts thereof. Heterologous cultures had a slower action than homologous microbes and material prepared from them. Only true W, *i.e.*, smooth, organisms underwent this change. The concept of the formation of individual Salmonella serotypes by loss variation is well known. According to this doctrine one has to suppose that all *S. typhi, S. paratyphi C,* as well as *Paracolobactrum Ballerup* 1 and 2 once harbored the Vi antigen. A reversal of the loss variation which led to the formation of pure W strains may be considered.

It is not possible, without testing enormous numbers of colonies, to be certain that Vi organisms are not present in small proportions avoiding detection when only a few thousand colonies are examined. In this case the addition of whole organisms or their fractions may supply materials favoring the multiplication of spontaneously occurring Vi forms, to such an extent that they establish themselves in competition to the W cells and become serologically demonstrable.

Since the activity of the transforming principle of pneumococcal types can be recognized at dilutions 5 x 10^7, it is possible that the activity of RNA, which was observed only in a few instances, was due to the presence of traces of DNA.

These experiments also indicate the close relationship of the Vi antigen of *S. paratyphi C* to that of *S. typhi* and *Paracolobactrum Ballerup.*

Summary. Heat-killed Vi organisms, the saline extracts of their acetone precipitates and DNA fractions induced W-V variation in *S. typhi, S. paratyphi* C and *Paracolobactrum Ballerup* strains.

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Inhibition of Steroid-Induced Adrenal Hypofunction. (21290)

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There is considerable evidence to support the view that the administration of adrenal cortical hormones suppresses adrenal function and induces adrenal atrophy in the rat(1-3). Clinical evidence indicates that patients on cortisone therapy also develop a functional and anatomical depression of the glands(4-8). Since it is generally agreed that the depressant effects of cortisone are the result of decreased secretion of ACTH by the anterior pituitary gland and that the adrenal hypofunction results from the lack of endogenous stimulation of the adrenal cortex, it appeared possible to employ a combination of cortisone and ACTH to eliminate or minimize the adrenal hypofunction induced by cortisone. Furthermore, it was felt that the ACTH would probably have a therapeutic action of its own through induced secretion of cortical steroids. Therefore, a study was undertaken to determine the effects of cortisone acetate and hydrocortisone on the adrenals of the intact dog and to determine whether concurrent ACTH administration would eliminate or minimize any adrenal hypofunction produced by the steroid hormones. The estimation of adrenal potential was made by observing the degree of eosinopenia evoked by test doses of ACTH before and after the course of treatment.

It was demonstrated that ordinary therapeutic doses of cortisone acetate and hydrocortisone in the intact dog cause a functional depression of the adrenal cortex which persists to some degree two weeks after cessation of treatment. Concurrent administration of ACTH lessens the steroid-induced adrenal depression, as evidenced by an earlier eosinopenic response in ACTH-steroid-treated animals than in animals receiving the steroid alone.

Materials and Methods. The animals used were laboratory acclimated male dogs 13 to 20 kg body weight at the beginning of the experiment. Eosinophil counts were determined by a modification of the technic of Spiers and Meyer(9). The counts were made on venous blood obtained without stasis and prevented from coagulating by addition of heparin, 50 units per ml of blood. *Adrenal response* to ACTH was determined on each dog in the following manner: (1) a blood sample was taken at 10:00 A. M.; (2) the "test dose" of ACTH, 0.5 USP unit per kg body weight in physiological saline, was injected intramuscularly immediately after the bleeding; (3) a second blood sample was taken four hours after the ACTH injection. The eosinopenic response was expressed by per cent difference between the zero-time count and the 4-hour count. An eosinopenic response of a 50% decrease in number over the pre-injection level was considered significant. The "test dose" response was carried out prior to and at intervals after cessation of treatment. Each dog received a single daily 0.5 ml intramuscular injection of the test preparation for a period of 28 days in the cortisone and cortisone-ACTH-treated animals and 21 days in the hydrocortisone and hydrocortisone-ACTH-treated group. Each ml of the preparations used contained the following:

Exp. #1
a) 10 units ACTH
  25 mg cortisone acetate aqueous susp.
b) 25 mg cortisone acetate aqueous susp.
c) 25 units ACTH
  25 mg cortisone acetate aqueous susp.

Exp. #2
a) 10 units ACTH
  25 mg hydrocortisone aluminum monostearate peanut oil gel
b) 25 mg hydrocortisone aluminum monostearate peanut oil gel

Exp. #3
a) 10 units ACTH
  25 mg hydrocortisone
  16% Knox Gelatin
  0.5% phenol
b) 10 units ACTH-pectate
  25 mg hydrocortisone
  16% Knox Gelatin
  0.5% phenol
c) 25 mg hydrocortisone
  16% Knox Gelatin
  0.5% phenol

Results. Exp. #1. It will be noted (Table I) that 3 of the 4 animals treated with the mix-

TABLE I. Eosinophil Response to ACTH of Dogs before and after Treatment with Adrenal Steroids Alone or in Combination with ACTH.

Exp. No.	Preparation and daily dose	Dog No.	% change of eosinophils in response to "test dose" of 0.5 u/kg ACTH															
			Pre-treatment	Days post-treatment														
				2	3	4	5	6	7	8	10	11	12	13	14	15	17	19
1a	Cortisone acetate, 12.5 mg; aqueous suspension; once daily/28 days	1	77	15					33						48			
		2	45	3					11						41			
		3	73	7					61						51			
		4	64	6					74						74			
1b	Cortisone acetate, 12.5 mg; ACTH, 12.5 units; aqueous suspension; once daily/28 days	5	61	45					30			49				57		
		6	85	56					61			81				54		
		7	37	6					15			23				44		
		8	78	58					53			80				54		
1c	Cortisone acetate, 12.5 mg; ACTH, 5 units; aqueous suspension; once daily/28 days	9	90	60					65						82			
		10	70	24					41						67			
		11	70	5					55						65			
		12	39	5											38			
2a	Hydrocortisone, 12.5 mg; aluminum monostearate peanut oil gel; once daily/21 days	13	68		30		4			1	3		44					45
		14	59		2		32			+70	10		11					24
		15	60		16		52			17	52							61
		16	88		31		37			51	38							40
		17	58		3		57			28	26							48
2b	Hydrocortisone, 12.5 mg; ACTH, 5 units; aluminum monostearate peanut oil gel; once daily/21 days	18	52		56		63											
		19	88		89		85											
		20	85		84		64											
		21	64		33		53			17	43							
3a	Hydrocortisone, 12.5 mg; 16% gelatin; once daily /21 days	22	65			82		15		29		20		53				
		23	63			50		32		41		73						
		24	63			8		34		35		42		42		51		
		25	57			44		41		16		36		42		57		
		26	38			12		12		25		23		30		35	48	
3b	Hydrocortisone, 12.5 mg; ACTH, 5 units; 16% gelatin; once daily/21 days	27	72			58		21		69		42		52				
		28	72			46		51		36		42		43				
		29	60			+5		25		26		25		26		31	33	35
		30	72			22		44		64		72		64				
		31	20			50		50		50		50						
3c	Hydrocortisone, 12.5 mg; ACTH-pectate, 5 units; 16% gelatin; once daily /21 days	32	61			43		19		40		42		41				
		33	50			55		53		56								
		34	55			57		+8		43		39		63				
		35	51			43		37		70		52		70				
		36	37			51		52		57		43		57				

ture had a normal response to the "test dose" of ACTH by the second day post-therapy. When the dose of ACTH was decreased to 5 units per day, the post-injection sensitivity of the ACTH-steroid-treated animal was inferior to that of the animal receiving the greater dose and was not different from the animal that received cortisone alone.

Exp. #2. Three of the 4 ACTH-hydrocortisone-treated animals showed a normal eosinopenic 'response 3 days post-injection, while none of the hydrocortisone-treated animals showed such a response. The adrenal depression of the hydrocortisone-treated animal persisted from 10 to 20 days after hormonal treatment.

Exp. #3. Animals were injected with hydrocortisone alone or in combination with ACTH, or ACTH-pectate, in 16% gelatin. Of the 5 animals that received hydrocortisone alone, 2 were not affected by the 21-day therapy, the other 3 showed depressed adrenal function. Although one of the 3 showed an adequate response to the "test dose" on the fourth day post-injection, such a response did not reoccur until the thirteenth day post-therapy. Complete adrenal recovery in these animals occurred by the fifteenth-seventeenth day post-steroid treatment.

Of the animals that received ACTH, or ACTH-pectate, with the hydrocortisone, 3 animals of each group showed an eosinopenia by the fourth day post-therapy, and complete recovery of all but one animal, #29, was obtained by the eighth day. Animal #29 never responded to the ACTH "test dose" which may have resulted from refractoriness to exogenous ACTH.

Discussion. It is apparent that the adrenals of the dog are rendered hypofunctional by exogenous adrenal steroid. This functional depression is not only reversible but appears to be prevented by the simultaneous injection of ACTH.

The adrenal response to ACTH after cortisone or hydrocortisone therapy is not unlike that described by Engleman *et al.*(10), who demonstrated in the human a delay in adrenal responsiveness to corticotropin during treatment with cortisone.

Although response of the dog adrenal gland to exogenous ACTH after simultaneous corticotropin-cortisone or hydrocortisone therapy supports the belief that the adrenal gland has a greater potential than the adrenal gland of the steroid-treated animal, definite evidence for the potential of the adrenal-pituitary axis is not now available. Experiments now in progress are designed to demonstrate whether the steroid-induced adrenal-pituitary axis lethargy can be prevented by simultaneous injection of ACTH with the steroid by subjecting the animals to a stress procedure after hormone treatment.

Summary. (1) Adrenocortical function of the dog is suppressed by administration of therapeutic doses of cortisone or hydrocortisone. (2) The suppression is prevented by simultaneous administration of ACTH.

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Blood, Urine, and Tissue Amylase in Depancreatized Rats. (21291)

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Amylase has been reported to occur in all tissues(1) and fluids(2) of animals which have been examined. The widespread occurrence of amylase in the body led to many early studies (3,4) on the site of production of the enzyme. Most of these studies concerned the pancreas, parotid glands, or the liver as the source of blood and urinary amylase. While it has been demonstrated that ligation of the ducts or experimental injury to the pancreas can produce an increase in the level of amylase in the blood and in its excretion in the urine, there is considerable disagreement on the effect of removal of the gland. Some workers, (5,6) have reported an increase in blood amylase, others(4,7) a decrease, and still others(2,8,9) no change following removal of the pancreas. The picture is further complicated by reports(10,11) that there is an initial decrease with a later irregular, but definite, return of blood amylase to control levels. However, the majority of investigators agree that amylase does not disappear from the serum as a result of complete pancreatectomy. Somogyi(12) reported that humans with various impairments in liver function exhibited subnormal amylase levels. This has been confirmed by others(13). In partially depancreatized dogs treated with chloroform (14) there is a distinct decrease in the level of blood amylase. Also low amylase values have been reported in acute alcoholism(15), a condition usually accompanied by liver damage. While the studies mentioned above suggest that the liver may be an important source of blood amylase, Somogyi(16) has reported that all the amylase in the liver is in the extracellular fluid and that the hepatic cells contain no amylase. However, unpublished studies (17) in this laboratory have shown that under certain conditions the liver may contain from 5 to 10 times as much amylase activity as can be accounted for by the blood in the organ.

A technic for complete pancreatectomy in the rat has been developed in this laboratory (18). In view of the lack of agreement among the studies on the origin and significance of blood and urinary amylase it seemed desirable to determine what changes occurred, if any, following pancreatectomy in the rat. The normal rat has a relatively high blood level and 24-hour urinary excretion of amylase, and has not been previously studied in this regard.

Methods. Adult rats of both sexes were housed in individual metabolism cages arranged for the quantitative collection of urine. All animals received the low residue diet, previously described(18), throughout the period of observation. The care of the animals and the operative technic have been described(18). The urine and blood samples were collected successively as follows: urine collected from 4 P.M. to 3 P.M. of succeeding day; food then removed and blood samples (0.1 ml of tail blood) collected 9-11 A.M. following morning. Food was then returned to the cage. The animals were sacrificed and tissue samples taken on the 30th day immediately after a fasting blood sample had been ob-

TABLE I. Amylase Levels in 8 Depancreatized Rats.

	Urine, unfasted		Blood, fasted	
	Mean	± S. D.	Mean	± S. D.
	Preoperative			
Controls*				
A	943†	379		
B	830	428	1386	249
C	716	334	1536	498
	Postoperative			
Days‡				
2, 3	764	364		
4, 5	827	279	1294	227
9, 10	891	355	1418	390
14, 15	1097	328	1379	425
19, 20	883	328	1493	364
24, 25	1118	357	1449	395
29, 30	1113	403	1765	623

* See text.

† Amylase units (19)/24 hr for urine; per 100 ml for blood.

‡ 1st number indicates day of urine collection; 2nd, day of blood collection.

TABLE II. Tissue Amylase Levels in Fasted Rats.

Tissue	Unoperated Mean*	Unoperated ± S. D.	Depancreatized Mean*	Depancreatized ± S.D.
Liver	30.1†	4.7	20.6	5.1
Kidney	7.3	1.3	6.0	1.2
Heart	6.8	2.8	4.3	1.0
Muscle	1.0	2.2	.9	.5
Duodenum	26.7	6.9	5.0	1.8
Jejunum	9.5	7.3	4.2	2.3
Spleen	4.5	5.2	7.4	11.0
Pancreas	25799.0	6848.0	53.7	35.8

* 8 animals.

† Amylase units (19)/g wet tissue.

tained. Unoperated control animals were run in parallel and tissue samples taken for comparison with the operated animals. Prior to removal of the pancreas three 24-hour urine samples and 2 blood samples were taken at 5-day intervals as described above and were used as control values for the operated animals. The levels of amylase activity in the blood, urine, and tissue samples were determined by a micromodification of the method of Smith and Roe(19). The data in the tables are given in terms of the "amylase unit" which is defined(19) as the amount of enzyme which will hydrolyze 10 mg of starch in 30 minutes to a stage at which no color is given with iodine at 620 mμ, under the conditions of the procedure.

Results. The data are shown in Tables I and II. Control levels for blood and urine and the tissue levels in unoperated rats agree closely with comparable data on normal rats obtained by Smith(17). Removal of the pancreas did not produce significant increase or decrease in the level of blood amylase or in the 24-hour excretion, either immediately or for 30 days following the operation. The absence of a change in urinary excretion after pancreatectomy is especially significant since it is usually assumed that the level of urinary amylase parallels the amylase activity of the pancreas. Kidney, heart, muscle, and spleen of depancreatized animals had essentially the same levels of amylase activity as normal rats. Sections of the small intestine in the depancreatized rats exhibited a definite decrease in amylase activity as would be expected. The residual amylase in the small intestine may be derived from the salivary glands. The pancreatic tissue indicated in Table II as analyzed for amylase in the depancreatized rats was the fragments of mesentery which normally contain the pancreas. The amylase activity of the liver was lower in the depancreatized rats than in the normal controls. This decrease can not be explained on the basis that liver amylase is in the blood contained in the organ inasmuch as the blood level was the same in the 2 types of animals. It is possible that a part of the liver amylase in the normal rat is derived from the pancreas or, more likely, that the depancreatized animals had the fatty livers, characteristic of such animals; this would lower the amylase level when calculated on a weight basis.

The finding that the amylase activity in the blood, urine, and tissues (except the liver and G.I. tract) of the rat is maintained at the same levels after pancreatectomy as before suggests that in the normal rat the pancreas is not an important source for the enzyme occurring in these fluids and tissues or that in response to removal of the pancreas other factors, *i.e.*, increased extrapancreatic production or diminished rate of destruction, compensate for the loss and thereby maintain the normal levels. The latter possibilities can not be evaluated at the present time but appear unlikely. These suggestions apply only to the role of the pancreas in the *normal* rat for, as reviewed above, the pancreas is an important source of the enzyme in blood and urine in conditions where the levels are elevated above normal. There is no conclusive evidence(16) that other tissues than the pancreas and parotid glands produce amylase. The literature(16) indicates that normally blood amylase does not originate either directly or indirectly in the salivary glands. However, during the 30-day postoperative period of observation each of the depancreatized rats excreted approximately 30,000 units of activity. From this it seems clear that some other organ or organs can produce appreciable quantities of amylase.

Summary. The levels of amylase in the blood, urine, and tissues of the rat were not significantly altered by pancreatectomy. It is suggested that some organ or organs, other than the pancreas, is the principal source of

blood, urinary, and tissue amylase in normal rats.

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Simultaneous Use of I^{131}-Albumin and Cr^{51}-Labelled Red Cells in Blood Volume Studies in the Goat. (21292)

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Several methods have been used to determine red cell and plasma volumes in various animals simultaneously(1-8). T-1824 dye and I^{131}-albumin are generally used for plasma labels. Frank and Gray(9) have recently reported the use of Cr^{51}-labelled plasma. Radioisotopes of P, Cr, and Fe are generally used for red cell labels. In many experiments here it was desirable to obtain plasma and red cell volumes simultaneously using labels which were available for other volume studies and which readily permitted several successive determinations. Small samples of blood for assay were also desired to reduce the blood loss due to frequent sampling. Most procedures require a considerable sampling loss where several volume determinations are made. The method of Gamble *et al.*(1), using I^{131} and P^{32}, although successful in the dog, was not used in the goat since goat red cells did not retain the phosphate.

In the method described below I^{131}-albumin and Cr^{51}-labelled red cells were used simultaneously. Hematocrit tubes were used for counting containers. Plasma was separated by centrifugation of blood and counted separately. The red cells were partially separated and counted. By correcting the cell plus plasma counts for the plasma counts, the red cell counts were obtained. The use of the hematocrit tube facilitates the separation of plasma and cells and requires no more than 1 cc of blood per sample. Volume determinations were made on normal goats using this method. Two *in vitro* recovery experiments were also performed.

Methods. Samples of blood were drawn from goats and the red cells were labelled with $Na_2Cr^{51}O_4$ as described by Gray and Frank (3). I^{131}-human serum albumin (Abbott) was diluted in isotonic saline to provide 0.5-1.0 μc/cc solutions. Wintrobe "EXAX" (Kimble Glass) hematocrit tubes were calibrated and used as containers for samples assayed for radioactivity. Volumes of samples were determined by reading the sample levels in the tubes. The hematocrit tube unit was used as the unit of volume. In order to avoid errors in counting due to changes in geometry an attempt was made to keep cell, standard solutions, and plasma levels approximately equal throughout an experiment. The level selected depends on the cell level as will be seen from the description of the method which follows. Blood samples were placed in hematocrit tubes and centrifuged for 30 minutes at 3000 r.p.m. A no. 17 hematocrit needle and syringe were used to remove all the plasma over the cells in the tube within a few units of the top of the cells. A very thin glass pipette was used to remove nearly all of the remaining plasma without disturbing the cells, leaving a fraction of a unit of plasma over the cells. The plasma thus removed was placed in another hematocrit tube at a level approximately equal to that of the cells from which the plasma was removed. Samples of standard solutions were similarly prepared in other hematocrit tubes. Volumes of red cells, white cells and remaining plasma were determined by reading their respective levels in the cell tubes. Volumes of plasma and standard solution samples were similarly obtained. Samples were counted in a well-type scintillation counter. Counts per unit volume (counts/volume) were calculated for standard solutions and plasma samples. The plasma counts/volume, volume of plasma remaining over the cells and the volume of plasma "trapped" in the cells were used to obtain the counts due to plasma (I^{131}) in the cell tubes. These counts were subtracted from the cell tube counts to obtain net red cell (Cr^{51}) counts from which counts/volume of red cells were determined. Hematocrit "trapped plasma" correction values (ratio of packed cell volume to packed cell and "trapped plasma" volume) were determined for each experiment using I^{131}-albumin. Since these values are large for goat blood they are particularly important in the determinations described below. About 10 cc of blood was taken from each source to be studied and a small drop of I^{131}-albumin (100-500 μc/cc) was added and mixed thoroughly. Three samples were placed in hematocrit tubes and centrifuged for 30 minutes at 300 r.p.m. Plasma and cells were separated as described above. Volumes of plasma, red plus white cells and plasma remaining above the cells were determined without correcting cell volumes for "trapped plasma." Cell and plasma samples were counted and the counts/volume were calculated for each as described above. The average counts/volume of plasma and cells were used to calculate the trapped plasma correction values: trapped plasma correction $= 1 - \frac{\text{counts/vol cells}}{\text{counts/vol plasma}}$. In order to check this method other methods were used simultaneously in some cases. *In vitro* plasma volume determinations were made using the dilution method with I^{131}-albumin and T-1824 dye (2,10,11). For some of these studies volumes were determined with I^{131} by counting 1 cc samples of plasma and standard solutions in plastic test tubes as well as in hematocrit tubes.

Except for the procedure described above the blood volume studies were essentially the same as those of others(1-10) using radioisotope dilution methods. Standard solutions were prepared by diluting 1 or 2 cc of Cr^{51}-labelled red cells and 0.5 or 1 cc of I^{131}-albumin solution in separate 100 ml volumetric flasks of isotonic saline containing 1 cc of carrier plasma and a small pinch of saponin. *In vitro* volume determinations were made by introducing 2 cc of Cr^{51}-labelled red cell suspension into 100 ml of blood, followed by 0.5 cc of I^{131}-albumin solution. Three samples each of blood and standard solutions were processed as described above. The average

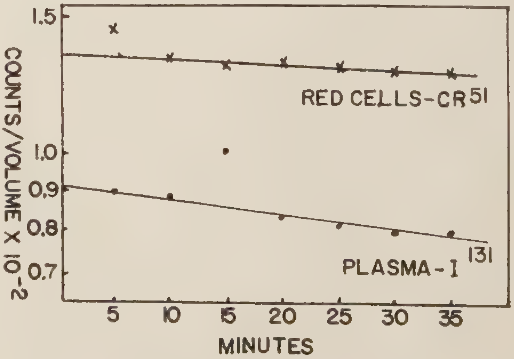

FIG. 1. Loss of Cr^{51} and I^{131} from blood.

TABLE I. Hematocrit "Trapped Plasma" Correction Factors Determined by Assay of I^{131}-Albumin Using the Direct Hematocrit Tube Method.

Observed hematocrit, %	Trapped plasma correction factor, %	Correct hematocrit, %
25.1	81	20.3
26.2	81	21.2
29.3	82	24.0
28.4	86	17.6
24.1	81	19.5
33.4	79	26.4
28.1	80	22.4
37.8	81	30.6
38.8	83	32.2
26.2	79	20.7
Mean	81	

counts/volume of red cells and plasma were compared to the corresponding average counts/volume of standard solution to determine the total plasma and red cell volumes in the flask. White cells were excluded from the determinations above but the volume of these in the cell tubes were averaged to obtain an estimate of the total white cells in the flask. Total blood volumes were obtained by adding the red cell and plasma volumes.

Four *in vivo* experiments were performed using the method described above. Seven to 35 cc of Cr^{51}-labelled red cell suspension and 2 to 10 cc of I^{131}-albumin solution were injected into goats through jugular catheters followed by a few cc of saline. Five 1 cc samples were taken in heparinized syringes between 10 and 30 minutes following the injection. A semilog plot of counts/volume of plasma and red cells was used to obtain values extrapolated to injection time (Fig. 1). The counts/volume at injection time were used to obtain plasma and cell volumes by comparison with counts/volume of standard solutions. Successive volume determinations were made at various times after the initial determination in 2 animals. In these cases 2 samples were taken just prior to injection. The counts/volume values were used to correct for activity already in the animal from previous determinations.

TABLE II. "Trapped Plasma" Correction Factors Using I^{131} and T-1824 Dye.

Hematocrit,* %	I^{131} hematocrit tube (direct)	I^{131} hematocrit tube (dilution)	I^{131} test tube (dilution)	T-1824 dye (dilution)
36.3	.84	—	.87	—
32.4	.81	.83	.87	.80
33.0	.81	.86	—	.82
31.8	.79	—	.81	.84
33.2	.83	—	.83	.93

* Uncorrected.

Results. "Trapped plasma" correction values (ratio of packed cell volume to packed cell and "trapped plasma" volume) determined by the methods described are given in Tables I and II. The mean value of ten determinations of different samples using the direct separation or hematocrit tube method was 81% and the range was 79-86%. There was good agreement among the direct and dilution methods (Table II). It is seen that the "trapped plasma" found in the goat is much greater than that reported for other animals(10-13). A "trapped plasma" correction value of 98% was found by the direct method described here for human blood which agrees with that found by others(10). "Trapped plasma" and corresponding hematocrit values are given in Table III for various centrifuging times. Changes in "trapped plasma" are seen to be related directly with changes in hematocrit.

TABLE III. "Trapped Plasma" Corrections and Hematocrits at Various Times of Centrifugation.

Centrifugation in min.	Uncorrected hematocrit, %	Trapped plasma corrections, %
30	27.8	82
60	25.1	90
90	24.6	90
120	24.2	92

The results of the *in vitro* volume experiments using Cr^{51} and I^{131} are given in Table IV. The differences in total blood volume and true volume in the two cases were 0 and 2%. White cell volumes (mean 1%) are not considered in total blood volume values so the deviations from the true values are augmented by a maximum of 1%, which increases the error in the first experiment and decreases it in the second. It is to be noted that not only accurate volumes but also accurate hematocrits were obtained in these experiments.

A summary of the *in vivo* experiments is given in Tables V and VI. The mean plasma volume was found to be 5.96% of body weight and that of red cell volume was 1.40% in 4 goats. The mean total blood volume of 7.37%

TABLE IV. *In Vitro* Volume Determinations Using I^{131} and Cr^{51} 100 cc Volumes.

Trapped plasma, %	Red cell vol, cc	Plasma vol, cc	Total vol, cc	White cell vol,* cc	Red cell vol / Total vol, %	Corrected hematocrit, %
18	24	76	100	1	24.0	24.0
19	21	77	98	1	21.4	21.2

* White cell vol estimate.

of body weight agrees well with values found previously(2) using Cr^{51} and T-1824 dye, which in 20 animals ranged from 5.68% to 8.94% of body weight. The rate of loss of I^{131} and Cr^{51} from the blood of the animals here is well within the range of values found by the authors in previous studies using these labels separately. The graph in Fig. 1 illustrates the curves used for calculation of volumes *in vivo*.

The results obtained from successive volume determinations in two animals at various times are given in Table VI. Animal A in which an increase in hematocrit occurred showed an increase in red cell volume also, while the plasma volumes were little changed. Animal B shows the reproducibility of results with only minor hematocrit changes.

The method of simultaneous volume determinations described here has obvious limitations. Hematocrit tubes must be accurately calibrated and must permit accurate reading of volumes. The maximum level of suspension or solution which can be used is dependent on the depth of the well of the crystal counter. In a given volume determination it is necessary that all tube levels be nearly the same in order to reduce variation in counting geometry. This becomes more important with red cell levels above the top of the well of the counter. To reduce this problem in animals which have much higher hematocrits it may be necessary to use smaller volumes of blood in hematocrit tubes or to use a counter with a deeper well. The relative amounts of I^{131} and Cr^{51} which are used are also important. Since the largest error may be in obtaining net Cr^{51} counts in packed cells it is desirable to use more detectable Cr^{51} than I^{131}. The amounts used will be determined by the counter used. In cases where large changes in hematocrit occur during the sampling period of a given determination some error may be encountered due to changing counting geometry.

Summary. A method is described for determinations of cell and plasma volumes using Cr^{51} and I^{131} simultaneously in which hematocrit tubes are used for counting and separation of cells and plasma. *In vitro* determinations of volumes of blood were within 1% of actual

TABLE V. Plasma and Cell Volumes in Goats Using I^{131} and Cr^{51} Simultaneously.

Wt, kg	Trapped plasma, %	Corrected hematocrit, %	Red cell vol, % of body wt	Plasma vol, % of body wt	Total blood vol, % of body wt
*28.6	19	24.9	1.36	5.44	6.81
27.3	18	20.0	1.49	6.14	7.63
28.1	14	17.6	1.04	6.42	7.46
30.0	21	22.3	1.73	5.84	7.57
Mean			1.40	5.96	7.37

* Male.

TABLE VI. Successive Volumes Using I^{131} and Cr^{51} Simultaneously in Goats.

Animal	Wt, kg	Date, time	Trapped plasma, %	Corrected hematocrit, %	Red cell vol, cc	Plasma vol, cc	Total vol, cc
A	30.0	3/31 0830	21	22.3	518	1750	2268
A	30.0	3/31 1130	20	26.5	588	1730	2318
B	28.1	4/15 1025	18	18.4	316	1405	1721
B	28.1	4/15 1445	18	19.0	304	1423	1727

volumes. Volumes and distributions found by this method in 4 goats are within the range of values found in the goat by other methods. Limitations of the method are also discussed.

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Effect of Various Methionine Analogues on Bacteriostatic Action of Methionine Sulfoximine.* (21293).

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In recent years 3 groups(1-3) working with agene (NCl_3) treated proteins have isolated and characterized the causative agent of running fits in dogs. This compound, methionine sulfoximine, is of particular interest not only as a convulsant but because it acts as a methionine antagonist. Reiner *et al.*(4) and Gershoff and Elvehjem(5) have prevented the symptoms of methionine sulfoximine toxicity in animals by the administration of adequate quantities of methionine. Gershoff and Elvehjem(6) have also concluded that the antimethionine activity of methionine sulfoximine is not specific for the enzyme systems directly involved in running fits but is general in nature. Methionine sulfoximine has also been shown to inhibit the growth of *Leuconostoc mesenteroides*(7), and this inhibition has been reversed by either methionine or glutamine (8,9).

The experiments to be reported here are the first of a series planned to investigate the biochemical and physiological mechanisms involved in the action of methionine sulfoximine. These investigations were made to determine the effect of various methionine analogs on the bacteriostatic action of methionine sulfoximine.

Experimental. These studies were carried out in 18 x 150 mm pyrex culture tubes using *Leuconostoc mesenteroides* P-60 as the test organism. All tubes contained 2 ml of modified Henderson-Snell media(10), containing 3.5 γ of DL-methionine per ml which is a suboptimal amount and allows approximately 50% of the maximum acid production. Acid production on this media with and without supplements of methionine sulfoximine and various methionine analogs was determined after incubation at 37°C for 68 hours. All determinations were made in sextuplicate.

Results. The results of these experiments are shown in Table I. These data, which were selected as being representative of a series of similar experiments, indicate that the ability of analogs of methionine to reverse methionine sulfoximine toxicity may or may not be related to their capacity to substitute for methionine in meeting the growth requirements of *Leuconostoc mesenteroides*.

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TABLE I. Effect of Methionine Analogues on Growth Inhibition of *Leuconostoc mesenteroides* P-60 by Methionine Sulfoximine.

Analogue used*	Methionine sulfoximine†	% of acid produced on unsupplemented media
0	+	49
DL-methionine	—	180
	+	166
3-methylmethionine	—	100
	+	53
S-methylpenicillamine sulfoxide	—	98
	+	48
3-methylmethionine sulfoxide	—	96
	+	48
Glutamine	—	93
	+	96
Methionine sulfoxide	—	178
	+	116
Methionine sulfone	—	93
	+	72
α-hydroxy-γ-methylmercapto butyric acid	—	137
	+	59

* 1.35×10^{-5} moles/tube.
† 6.75×10^{-7} moles/tube.

3-methylmethionine, S-methylpenicillamine sulfoxide, and 3-methylmethionine sulfoxide were without effect under the conditions of this experiment.

Methionine sulfoximine inhibition was completely reversed by glutamine; however, glutamine showed no growth promoting activity in the absence of the antagonist and hence cannot replace methionine for the growth of this organism. Similar studies with asparagine and citrulline were negative.

Methionine sulfoxide can serve as a source of methionine for *E. coli*(11), *L. arabinosus* (12), and *S. faecalis*(13). In these experiments *Leuconostoc mesenteroides* utilized methionine sulfoxide to the same degree as methionine in the absence of methionine sulfoximine, but methionine was more effective than the sulfoxide in reversing the inhibitory effect of the antagonist.

Methionine sulfone does not serve as a source of methionine for *L. arabinosus*(12) or *S. faecalis*(13). Similarly it was inactive for *Leuconostoc mesenteroides* as a source of methionine but appeared to reverse partially the toxicity of the antagonist.

α-hydroxy-γ-methylmercapto butyric acid has been reported to be equivalent to methionine in meeting the nutritional requirements of rats and chicks(14). This hydroxy analog of methionine was found to have considerable but not equivalent methionine activity for *Leuconostoc mesenteroides.* Thus it was unexpected to find that it was perfectly ineffective in reversing methionine sulfoximine toxicity.

Summary. A number of methionine analogs were studied for their ability to replace methionine and reverse methionine sulfoximine induced growth inhibition. Relative activity in replacing methionine and reversing methionine sulfoximine toxicity are not necessarily correlated. Some compounds which counteract methionine sulfoximine toxicity have no methionine-like activity.

The author wishes to thank Doris Hedrick and Ruth Sullivan for their technical assistance, the Monsanto Chemical Co. for supplying the α-hydroxy-γ-methylmercapto butyric acid and Dr. L. Reiner for synthesizing all of the other analogues used in these experiments.

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Effect of Soybean Sterols on Liver Deposition of Cholesterol-C^{14}. (21294)

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(Introduced by Herald R. Cox.)

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The prevention of hypercholesterolemia in cholesterol-fed chickens(1,2) and rabbits(3) has been successfully accomplished by the concomitant feeding of soybean sterols. This effect has been explained as an interference phenomenon, the plant sterols somehow altering absorption of cholesterol(3). Using cholesterol-4-C^{14} in rats, Hernandez *et al.*(4) demonstrated that either mixed soybean sterols or β-sitosterol resulted in a marked decrease in the amount of cholesterol recovered from thoracic duct lymph. This finding has recently been challenged by Rosenman and coworkers(5) who found no reduction of cholesterol absorption into the thoracic duct lymph in a similar experiment. The latter authors, who fed 100 mg of cholesterol, suggest that the difference is due to the amount (4 mg) of labeled cholesterol administered by Hernandez. This amount is supposedly less than the average amount of cholesterol ingested daily by rats on a stock diet.

We have completed experiments involving feeding of labeled cholesterol to rats on various sitosterol regimens with a view to gauging the effect of the soy sterols on liver storage of the marked sterol as well as on fecal excretion of labeled material. The results would indicate that a large excess of soybean sterol may, indeed, reduce liver deposition of ingested cholesterol. These results tend to substantiate the findings of Hernandez *et al.*

Experimental. Three groups of 4 male Wistar strain rats (500 g) were used. Groups 1 and 2 were maintained on normal rat chow (Wayne Rat Pellets, Allied Mills, Chicago, Ill.) augmented with 3% cholesterol in 4% corn oil for 120 days. Group 3 was maintained on rat chow augmented with 3% cholesterol plus 5% mixed soy sterols (Distillation Products, Rochester, N. Y.) in 4% corn oil for the same period. Each rat was then given about 10 mg random-labeled cholesterol-C^{14}(6) in one cc of corn oil by stomach tube. In the case of Group 2, the cholesterol was followed immediately by one g of mixed soy sterols in corn oil. All animals were maintained in individual metabolism cages for 4 days; the feces and urine of each animal being pooled and assayed for carbon-14 activity. After 4 days the rats were sacrificed and the livers removed, dissolved in warm alcoholic KOH and the solution separated into non-saponifiable and acidic fractions, each of which was assayed for radioactivity. Radioactivity measurements were made in a windowless gas-flow counter using infinitely thick plates of aliquots of the solutions obtained.

Results. Data are given in Table I.

Discussion. Inspection of the average values reveals that while the total activity recovered for the 3 groups was approximately the same, its distribution differed widely. The dose of administered cholesterol-C^{14} was small compared with the dose of cholesterol which the animals had been receiving, so that one cannot argue that the labeled cholesterol represented a unique dietary addition. The soy sterols evidently interfere with the adsorption of cholesterol but whether the mechanism is a competition for sites of esterification or a mechanical mixing which decreases cholesterol availability is not clear from these data. It is interesting to note that it is the amount of non-saponifiable material in the liver that differs, the activity recovered in the acidic fraction being about equal for the 3 groups.

The dose of soy sterols required to reduce the amount of cholesterol deposited in the liver after a single feeding of a small quantity of cholesterol appears to be rather large, since in this experiment even a hundredfold excess of soy sterol (Group 2) did not fully inhibit liver storage of cholesterol-C^{14}. In the animals fed 5% soy sterol with their diet for 3 months (Group 3) liver deposition of labeled choles-

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* Deceased.

TABLE I. Per Cent Administered C^{14} Recovered after Feeding Cholesterol-C^{14}. Three groups.

Regimen	Rat No.	Dose chol.-C^{14} (mg)	Liver		Feces		Urine	Total
			NS*	Acid	NS	Acid		
3% cholesterol (120 days) then cholesterol-C^{14}	1	9.4	5.14	.54	16.74	.71	ND†	23.13
	2	9.4	7.82	.78	24.19	1.58	.04	34.41
	3	9.0	2.74	.49	16.16	.43	ND	19.82
	4	10.3	10.75	.28	23.47	1.08	"	35.58
Avg			6.61	.52	20.14	.95	.01	28.24
3% cholesterol (120 days) then cholesterol-C^{14} and 1 g soy sterol	5	11.1	1.60	.44	26.45	1.69	.03	30.21
	6	9.6	.87	.13	23.62	1.38	.05	26.05
	7	9.1	4.55	1.10	32.69	2.03	ND	40.37
	8	13.1	.18	.57	17.77	.84	"	19.36
Avg			1.80	.56	25.13	1.49	.02	29.00
3% cholesterol + 5% soy sterol (120 days) then cholesterol-C^{14}	9	11.5	lost	.72	32.90	1.10	.08	—
	10	10.0	.02	.79	25.55	2.74	ND	29.10
	11	10.2	.89	1.01	34.10	1.91	"	37.91
	12	13.1	.50	ND	18.49	1.98	"	20.97
Avg			.47	.63	27.76	1.93	.02	30.81

* NS = non-saponifiable. † ND = no detectable radioactivity.

terol was still less than that in Group 2, but no estimate of the amount of soy sterol which may have been present in the gut of these animals has been made.

In work with humans, Pollak(7) reported that 10 g of sitosterol daily lowered serum cholesterol in his subjects; on the other hand, Wilkinson *et al.*(8) have found that doses of sitosterol as high as 60 g/day do not reduce serum cholesterol levels. The timing of the sitosterol feedings may have some bearing on these discrepancies.

Summary. 1. The effect of soybean sterols on liver deposition and fecal excretion of cholesterol-C^{14} following a single feeding of the labeled material has been studied using cholesterol-fed rats. 2. Average recoveries of labeled cholesterol in liver (measured as radioactivity recovered from liver non-saponifiable fraction) were 6.61% in a cholesterol-fed group, 1.80% in a cholesterol-fed group receiving one g of soy sterol immediately after cholesterol-C^{14} administration and 0.47% in a group fed both cholesterol and soybean sterol for 120 days. 3. Average recoveries of radioactivity in the non-saponifiable fraction of feces were 20.14%, 25.13%, and 27.76%, respectively, for the 3 groups described above.

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Regulation of Connective Tissue Hexosamine Levels by the Anterior Pituitary and Thyroid Glands. (21295)

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Hexosamines* are important constituents of connective tissue ground substance and are distributed largely in the mucopolysaccharides (1) and the plasma proteins of connective tissue(2). The concentration of hexosamines is increased in the orbital connective tissue of guinea pigs rendered myxedematous by either thyroidectomy alone, or by the administration of thyrotropic hormone to the thyroidectomized animals(3). In order to study further the physiological factors concerned with the production of these hexosamine changes, similar studies have been made in the rat. Although the response of the rat is shown to be less sensitive than that of the guinea pig, the levels of hexosamines in rat orbital connective tissue may be also controlled by the thyroid and the anterior pituitary glands.

Methods. Male Sprague-Dawley rats were individually caged, fed a stock diet(4), and permitted to drink water *ad libitum.* Food intake was not measured since it has been shown that wide variations in food intake will not influence the concentration of connective tissue hexosamine(5). Animals of approximately the same weight were used for each experiment. All operations were performed under ether anesthesia. The rats were hypophysectomized by the parapharyngeal approach. The hypophysectomized animals and their controls received daily subcutaneous injections of 4000 units of penicillin. All hormone preparations were administered subcutaneously in single daily doses. The thyrotropic hormone preparation (TSH)† was formulated in corn oil with 2% aluminum monostearate. Thyroxin was made up in water adjusted to pH 9, and the growth hormone (GH)‡ preparation was dissolved in water. All the animals were killed by exsanguination under ether anesthesia by cutting a carotid artery. Completeness of hypophysectomy was checked post-mortem by examining the sella turcica under magnification. Completeness of thyroidectomy was checked in one group by the radioautographic technic of Reinhardt(6).§ In this paper, if the mean area of the images on the two roentgen films was less than 14 mm^2, the rat was considered to be completely thyroidectomized or to have a trace. If the mean area was greater than 14 mm^2 the rat was considered to be incompletely thyroidectomized. *Orbital connective tissue* was obtained after removing the eyelids flush with the orbital rims and separating the eyeballs from the conjunctiva, extra-ocular muscles and optic nerves. The remaining orbital tissues were removed; the Harderian gland was separated, leaving a mass which consisted of loose orbital connective tissue, the extra-ocular muscles, and a small amount of scleral conjunctiva. We have referred to this entire mass as orbital connective tissue since the conjunctiva and muscles make up only a small fraction of the total mass. For chemical analyses, the connective tissue of both orbits was pooled. After weighing, all the samples were placed in an oven at 101-104° C for 18 hours, re-weighed, then extracted in a Soxhlet apparatus for 3 hours with petroleum ether (B.P. 60-70° C). The dry, fat-free samples were weighed and the water and fat contents calculated. Water is expressed as per cent $\frac{(\text{Wet Wt-Dry Wt}) \times 100}{\text{Wet Wt}}$. The fat content of orbital connective tissue rarely exceeded a range of 1-3% and was not influenced by any of the physiological situations studied.

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* The naturally occurring hexosamines are glucosamine and galactosamine. Method used here does not distinguish between these amino sugars.

† Armour thyrotropic hormone, Lot No. 129-31PRR3 was kindly supplied by Dr. Irby Bunding, Armour Laboratories, Chicago.

‡ Growth hormone, No. C_5I_5, E. R. Squibb & Co., was kindly supplied by Dr. Robert W. Bates. On bio-assay, this preparation at a dose level of 160 μg/day, induced a weight gain of 1 g/day in 100 g hypophysectomized rats.

§ We are indebted to Dr. Robert O. Scow for preparing the radioautographs.

These data are therefore not included. *Hexosamine* determinations were made on the dry defatted tissues. After a preliminary hydrolysis in 4 N hydrochloric acid for 15 hours, hexosamines were separated on Dowex-50, and determined by a modification of the method of Elson and Morgan(7). Hexosamine values are expressed as micrograms present in total orbital connective tissue and as milligrams per 100 g dry defatted tissue. All values are reported as Mean ± Standard Error.

Results. Thyroidectomy. To study the effect of thyroidectomy on the composition of orbital connective tissue, 3 groups of rats were used, 1) a control group (9 rats) sacrificed at 28 days of age, 2) a control group (10 rats) sacrificed at 76 days of age, and 3) a group (8 rats) completely thyroidectomized at 28 days of age and sacrificed at 76 days of age. The thyroidectomized group had a marked retardation of body growth (94 ± 5 g vs. 234 ±6 g for the 76-day, and 60 ± 2 g for the 28-day controls). The relative amount of connective tissue (mg/100 g Body Wt) did not change following thyroidectomy (73 ± 4 vs. 70 ± 1 for the 28-day controls). Whereas in the growing controls this ratio decreased (54 ± 2). Following thyroidectomy, the concentration of water (79.2 ± .3%) and hexosamine (494 ± 12 mg/100 g dry wt) remained the same as the 28-day controls (79.6 ± .4% and 493 ± 14 mg/100 g), whereas in the growing controls the concentrations of water (77.3 ± .3%) and hexosamine (384 ± 5 mg/100 g) decreased significantly.

In order to examine the hormonal factors responsible for this inhibition of connective tissue growth and development, 22 rats, weighing 110-130 g were used. They were divided into 5 groups, 1) an intact control group, 2) an uninjected thyroidectomized group, and thyroidectomized group receiving, 3) 3 μg of thyroxin, 4) 3 μg of thyroxin plus 500 μg of growth hormone and 5) 500 μg of growth hormone. The growth hormone preparation (C_5I_5) contained thyrotropic activity. All the animals were treated daily for 7 days from the day of operation. The differences in these data are not as striking as in the previous experiment, since the time elapsed was shorter and the rats were not screened for "completeness" of thyroidectomy. The concentration of hexosamine (mg/100 g dry wt) was again higher in the uninjected thyroidectomized group (448 ± 1) than in the control group (392 ± 10). This inability of thyroidectomized rats to develop the decrease in hexosamine concentration seen with normal growing rats was overcome by the administration of thyroxin, with (384 ± 1) or without growth hormone (393 ± 17) but not with growth hormone alone (458 ± 13). The weight of the connective tissue or its water content was not significantly altered in any group.

Hypophysectomy. In order to interpret the changes seen following thyroidectomy, the effects of growth hormone, TSH and thyroxin were studied in hypophysectomized rats. In the first experiment 3 groups of rats, with 6 rats in each group, were used, 1) an intact control group, and 2 groups of hypophysectomized rats, 2) uninjected and 3) injected daily with 2.5 μg of thyroxin from the day of operation. All were sacrificed 7 days from the day of operation. In comparison with the intact controls, the hypophysectomized rats had a significant retardation of body growth (99 ± 4 g vs. 135 ± 6 g) and connective tissue growth (62 ± 2 mg vs. 74 ± 4 mg) but no change in water (79.8 ± .3% vs. 79.6 ± .4%), or the concentration of hexosamine (406 ± 10 vs. 407 ± 16 mg/100 g dry wt). Thyroxin in the small maintenance dose given had no effect on the composition of connective tissue.

In the second experiment (Table I) groups of hypophysectomized rats with 3 or 4 rats in each group received daily injections of, 1) 24 μg of thyroxin, 2) 2 mg of thyrotropic hormone, and 3) 2 mg of growth hormone, known to contain thyrotropic activity. All rats were treated daily for 7 days following the day of operation and sacrificed on the 8th day. Thyroxin, even in the toxic doses administered, failed to produce any significant alterations in the weight, water, or hexosamine content of connective tissue. Both pituitary preparations produced significant thyroid gland hypertrophy. Thyrotropic hormone had no significant effect on the amount or concentration of connective tissue hexosamine or water. Growth hormone, on the other hand, stimu-

TABLE I. Effect of Administration of Thyroxin and Pituitary Hormones on Orbital Connective Tissue of Hypophysectomized Rats.

			Orbital connective tissue			
					Hexosamine	
Group	Body wt (g)	Thyroid (mg)	Wet wt (mg)	mg/100 g body wt	Total μg	mg/100 g dry wt
Control	100 ± 3	6.2 ± .1	50 ± 2	50 ± 2	41 ± 3	441 ± 18
Thyroxin	96 ± 5	—	52 ± 1	54 ± 3	42 ± 1	413 ± 17
TSH	104 ± 8	11.1 ± 1.0	58 ± 4	56 ± 3	48 ± 2	433 ± 6
GH	135 ± 2	10.9 ± 1.2	74 ± 2	54 ± 2	61 ± 2	457 ± 4

lated body growth with a proportionate increase in the weight of orbit connective tissue and the total amount of hexosamine. The concentration of hexosamine was not significantly increased. No group showed any significant alteration in the concentration of water.

Discussion. It has been demonstrated in the guinea pig that thyroidectomy produces orbital myxedema with an increase in the concentration of connective tissue hexosamine (3). An even greater increase results when thyroidectomized guinea pigs are given pituitary extract (crude thyrotropin).

In the growing rat the concentration of orbital connective tissue hexosamine gradually decreases. Similar observations on age changes have been made on subcutaneous tissue of the rat(5) and in rat skin where the decrease in the concentration of hexosamine is shown to result primarily from a relative increase in collagen(8). The normal decrease seen with growth in the concentration of orbital connective tissue hexosamine is prevented by thyroidectomy, but can be restored by the administration of thyroxin in small doses. On the other hand, the normal decrease in the concentration of hexosamine with growth is not abolished by hypophysectomy, and thyroxin in small or large doses will not influence the concentration of hexosamine. Growth hormone, however, when administered to the hypophysectomized rat, in addition to increasing body weight, will increase the weight of connective tissue and the total hexosamine proportionately. Thyrotropic hormone had no effect on the amount or concentration of hexosamine in the hypophysectomized rat.

Rat orbital connective tissue is less sensitive than that of the guinea pig(3) to the effects of thyroidectomy and administration of pituitary hormones. It is apparent, however, that even rat orbital connective tissue can be influenced by the thyroid and by the anterior pituitary gland. A balance must exist between the production of growth hormone and thyroid hormone which determines the amount of hexosamine and connective tissue present in the orbit.

Summary. 1. A decrease in the concentration of hexosamine in orbital connective tissue is observed with increasing age in normal rats but is abolished following thyroidectomy. It can be reversed, however, by the administration of thyroxin. The concentration of connective tissue hexosamine in hypophysectomized rats is similar to controls of the same age and is unaffected by either small or large doses of thyroxin. Growth hormone, on the other hand, produces an increase in the amounts of connective tissue and hexosamine with no change in the concentration of hexosamine. 2. It is suggested that the concentration and amount of hexosamine in orbital connective tissue in the rat is determined by a balance between the production of hormones of thyroid and anterior pituitary (growth hormone). 3. The response of rat orbital connective tissue to thyroidectomy or to the administration of pituitary extract appears to be less sensitive than that in the guinea pig.

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Effects of Administering Various Blood Serum Constituents on Gamma Globulin Levels of Baby Pigs.*† (21296)

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(Introduced by G. Howard Satterfield.)

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Nutritional investigations with baby pigs that have been permitted to nurse their dams have emphasized the need for means of starting pigs without relying on the dams' colostrum. Fat studies at the North Carolina Agricultural Experiment Station which were initiated with pigs which had nursed 2 days revealed considerable variation in pig response. The quantity and quality of colostrum consumed by the pigs were considered to be an important factor in this variation. The need for means of protecting newborn pigs from disease was recognized and some success has been obtained by conducting studies in isolated quarters. However, the results have been too variable from experiment to experiment to be reliable and pigs successfully started under isolated conditions have exhibited a high mortality rate when placed in contact with swine in a natural environment.

Accordingly, an investigation was undertaken to study possible means of providing the protection to newborn pigs which is normally obtained through the dams' colostrum. This report covers investigations on the effects of administering lyophilized bovine and porcine serum solids and porcine gamma globulin on the blood serum gamma globulin content of newborn pigs.

Procedure. Investigations were conducted with newborn pigs removed from the mothers at birth. After the completion of farrowing, the pigs were identified by ear notches, weighed, and the pigs from a litter were randomly assigned to the experimental treatments on the basis of weight. Blood samples for electrophoresis were collected after the pigs had been on the treatments for 24 hours. A minimum of 4 replications were used in each trial and when additional pigs from a litter were available they were assigned to one of the treatments. Difficulty in obtaining an adequate quantity of blood from a few of the pigs made it impossible to obtain data on the total number of animals started in each trial. Eight different treatments were used in the first experiment: 1) 180 ml of sows' colostrum, 2) 120 ml of cows' colostrum, 3) 10 g, and 4) 20 g of lyophilized bovine serum solids, 5) 10 g, and 6) 20 g of lyophilized porcine serum solids, 7) fasted, and 8) permitted to nurse at will. The materials listed for the first 6 treatments were administered by stomach tube in 4 equal portions at 6-hour intervals. The quantities of cows' and sows' colostrum fed were calculated to provide approximately the same quantities of protein in the decaseinated portion of the colostrum. The serum solids were diluted in water to a concentration of 10 g of serum solids to 40 ml of water. In the second experiment the treatments consisted of: 1) porcine gamma globulin administered intraperitoneally, 2) porcine gamma globulin administered orally in cows' milk, 3) bovine serum solids administered orally in cows' milk, and 4) controls which received cows' milk only. The porcine globulin solution used contained 10% gamma globulin, and 20 ml were used for both the parenteral and oral administration. For oral administration the 20 ml of gamma globulin solution were added to 130 ml of milk and fed with a bottle and nipple in 6 equal feedings at 4-hour intervals. A total of 7.5 g of bovine serum solids was fed each pig receiving this treatment. The 7.5 g of solids were dissolved in 20 ml of water, and

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† The lyophilized bovine and porcine serum solids were provided and electrophoretic patterns were obtained through the courtesy of Dr. Chris. P. Segard of the Wisconsin Alumni Research Foundation. Porcine gamma globulin and amorphous trypsin inhibitor were provided by The Armour Laboratories through the courtesy of Dr. M. A. Schooley.

TABLE I. The Influence of Fasting, Normal Nursing and Administration via Stomach Tube of Sows' Colostrum, Cows' Colostrum, Bovine Serum Solids and Porcine Serum Solids on the Blood Serum Gamma Globulin Content of One-Day-Old Pigs. (Each value represents percent of the total protein as gamma globulin for an individual animal.)

		Administered by stomach tube					
				Porcine serum solids		Bovine serum solids	
Fasted	Nursed dams	Sows' colostrum	Cows' colostrum	10 g	20 g	10 g	20 g
0	46	18	3	0	5	0	4
0	43	25	0	3	6	0	3
0	41	11	0	4	0	14	5
0	45		0	4	7	0	0
				5	8	0	3

this was added to 130 ml of milk for feeding as indicated above. The control pigs and those receiving gamma globulin parenterally were fed 150 ml of cows' milk. The gamma globulin was injected intraperitoneally in 6 equal portions at the time the pigs were fed. The cows' milk used had been pasteurized and homogenized. In the third experiment bovine serum solids and porcine gamma globulin were compared when fed orally with and without a source of trypsin inhibitor. (Laskowski and Laskowski(1) reported that bovine colostrum contains large amounts of trypsin inhibitor.) Porcine gamma globulin was also administered parenterally. The procedures for oral feeding were the same as in the second experiment. Amorphous trypsin inhibitor was used at the rate of 750 mg to 150 ml of each milk preparation fed.

All pigs were bled 4 to 5 hours after the last feeding or treatment was given. Approximately 20 ml of blood were drawn from the anterior vena cava with a hypodermic syringe and needle as described by Carle and Dewhirst (2). The blood was permitted to stand at room temperature 2 or 3 hours and was then placed in a refrigerator for 10 to 12 hours to permit retraction of the clot. Ten ml of serum were pipetted off, sealed in vials and frozen. The frozen samples were packed with ice in an insulated container and shipped via express to the laboratory for electrophoresis.‡

Results. The results of the first experiment are shown in Table I. The only consistent response in the serum globulin levels was obtained with the pigs which nursed or were administered sows' colostrum via stomach tube. The cows' and sows' colostrum which were used represented pooled collections from 3 animals. It had been preserved in a frozen condition for approximately 3 weeks. The lower gamma globulin values for the pigs administered sows' colostrum by stomach tube may have been due to a lower intake (the pigs left with the sows nursed *ad libitum*), a lower gamma globulin content of the colostrum used, or to method of administration. The gamma globulin values for the nursing pigs are slightly higher than those reported by Foster *et al.* (3). The porcine and bovine serum solids and cows' colostrum did not cause a marked or uniform response in the serum gamma globulin levels.

The results of the second and third experiments are included in Table II. Three of the treatments were common to each experiment. The porcine gamma globulin failed to give a significant response when fed orally with cows' milk, but when it was administered intraperitoneally the serum gamma globulin levels approached the levels obtained previously with nursing pigs. The inclusion of trypsin inhibitor in the milk-gamma globulin mixture did not improve the serum gamma globulin levels. As in the first experiment, oral administration of bovine serum solids did not cause a significant response and inclusion of trypsin inhibitor did not affect the results.

Discussion. Inasmuch as porcine gamma globulin caused a marked response in serum gamma globulin levels when administered in-

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‡ The determinations for the first experiment were made under the direction of Dr. J. W. Palmer of Squibb Laboratories and for the second and third experiments under the direction of Dr. Kurt G. Stern of the Polytechnic Institute of Brooklyn.

TABLE II. Influence of Porcine Gamma Globulin and Bovine Serum Solids Administered by Different Procedures on Blood Serum Gamma Globulin Content of One-Day-Old Pigs. (Each value represents % of total protein as gamma globulin for an individual animal.)

Controls	Porcine gamma globulin			Bovine serum solids	
Cows' milk	Orally	Orally with trypsin inhibitor	Parenterally (i.p.)	Orally	Orally with trypsin inhibitor
0	0	0	35	9	7
0	6	9	29	0	0
0	0	5	44	0	0
0	0	0	40	0	5
	9		27	0	
	0		41	2	
	0		37	8	
			31	6	
			16	0	
				3	

traperitoneally but not when administered orally it is evident that processes related to the alimentary tract prohibited gamma globulin from reaching the blood stream. This indicates that sows' colostrum contains a component(s) that enables the gamma globulin to be absorbed from the alimentary tract without being digested. Evidence accumulated in this work indicates that trypsin inhibitor alone is not responsible and that the component(s) is not present in lyophilized serum solids in effective amounts. The failure of cows' colostrum to give a response with baby pigs suggests a species difference in the colostrum of the pig and the cow or in factors affecting the absorption of gamma globulin by the pig and the calf.

Summary. Data have been presented which show that porcine gamma globulin, porcine and bovine serum solids and cows' colostrum do not cause a marked or consistent response in the serum gamma globulin level of one-day-old pigs when administered orally. However, parenteral administration of porcine gamma globulin gave a response which approached that obtained with nursing pigs. The inclusion of trypsin inhibitor with orally administered porcine gamma globulin and bovine serum solids did not affect the serum gamma globulin level.

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L Colonies from Hemolytic Streptococci: New Technic in the Study of L Forms of Bacteria.*† (21297)

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The peculiar, small, fragile growth form usually designated as an L form has been isolated previously from alpha hemolytic streptococci(1). L forms from this species were obtained using technics successful in other species, in that of a large number of strains exposed to penicillin on horse serum agar plates, a few yielded L type colonies. Using these methods L type colonies have not been obtained from beta hemolytic streptococci, although many attempts have been made in this laboratory in the past several years, and during the past winter alone we have examined between 50 and 100 strains.

Results. Positive results have been obtained

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only after much experimentation with various media. The decisive factor has proved to be very simple, namely the concentration of electrolyte. Thus of 5 strains of group A streptococci that have been tested repeatedly on .25 M to 1.1 M NaCl plates, all have repeatedly produced L type colonies, although with varied regularity. Of 21 other strains of beta hemolytic streptococci, most of which are group A, that have been tested a single occasion on .5 to .58 M NaCl plates, 5 strains have produced L colonies. The optimal concentration of salt has not been determined but L type colonies have been observed in one strain with .25 M NaCl and in several strains with 1.1 M NaCl; transition to L forms has not been observed to occur at concentrations of NaCl below this, when other electrolytes were present in low concentrations, and NaCl has not been tested in higher concentrations. Several other salts have also been found to favor the development of the L colonies, including KCl, NH_4Cl, $CaCl_2$, $MgCl_2$, and Na_2HPO_4. Wide ranges in concentration of these electrolytes have not been studied, but L colonies have been observed on plates containing as little as .18 M of the bivalent cations and .14 M Na_2HPO_4.

Increased salt concentration favors the production of L forms not only from the streptococci but also from other species. This has been observed with the colon bacillus. Although scanty L growth of *E. coli* has been observed previously(2), a sufficiently abundant growth of these L forms has been obtained to permit successful subculture of the L forms for the first time by increasing the salt concentration of an otherwise appropriate medium.

Another indication of the importance of the salt concentration in the cultivation of L forms has been observed with a strain of alpha streptococci that has been carried in this laboratory for more than a year as a known L former. With this strain it has been possible to devise a medium in which all elements are chemically defined except for horse serum and agar. With this system it has been found that the concentration of electrolyte is critical for the successful transformation to the L form, but the required concentration is of a much lower order than with the beta streptococci, and is within the range usually employed in bacteriological media.

The technic with which L forms were isolated from beta hemolytic streptococci is as follows. The appropriate concentration of electrolyte was incorporated in plates containing a tryptic digest of beef heart infusion as base, 1.3% agar, and 10% horse serum inactivated at 56°C for 30 minutes. The plates were heavily inoculated with an 18-24 hour bacterial growth and about 1000 units of penicillin was deposited in a small trough cut in the agar. The plates were incubated at 36°C anaerobically, using the Fortner biological method. After a few days L type colonies appeared. These developed most regularly at the margin of the area where bacterial growth was inhibited.

The transition into L forms occurred in the same way as in other species. Some of the cocci increased greatly in size producing round or polygonal forms of 10-20 μ. The L growth started from these. (See photographs.) With .08-.16 M NaCl exposure to penicillin irregularly produces some pleomorphism with round forms 3-5 μ occurring but without further development. With .25 M NaCl one of 3 strains examined produced many large bodies and infrequent L colonies. Although observations are yet limited, transition into the L form has not been observed in the absence of anaerobic incubation, but growth on subculture is satisfactory with aerobic incubation.

L colonies develop on the high salt plates with high concentrations of penicillin (1000 U/cc or more) incorporated in the media but growth is less regular and less abundant than with the technic described above. After transition to the L form has occurred growth on subculture is not influenced by high concentrations of penicillin in the media.

The L forms isolated from the group A streptococci are similar both in the gross appearance of the colonies and in the microscopic morphology to the L forms of the alpha streptococci and present in every respect the characteristic properties of L forms. The original strains of hemolytic streptococci have been regained from the L forms of 2 strains. This was accomplished on

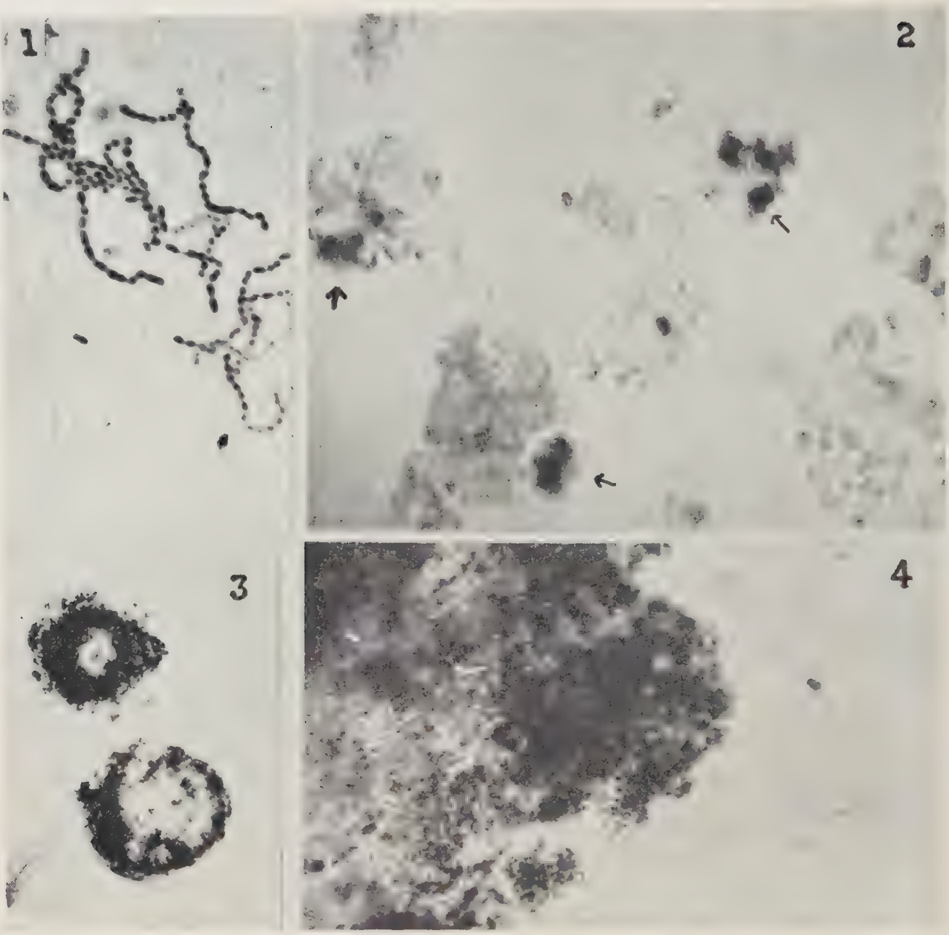

FIG. 1. Group A hemolytic streptococcus grown on plain agar, magnified 900×.

FIG. 2. Group A streptococcus exposed to penicillin on salt medium. The cocci are swollen to large bodies and several small L colonies (marked with arrows) are developing. Magnification 100×.

FIG. 3. Large L colonies of group A streptococcus magnified 100×.

FIG. 4. The edge of an L colony magnified 900×. The spreading growth is into the agar. (Photographs 1 and 2 were made from wet preparations; 3 and 4 from dried preparations.)

.58 M NaCl plates containing no penicillin, the plates being incubated aerobically. The inocula were from the fifth serial transplants of the L form on plates containing high concentrations of penicillin. The serological properties of the L forms of the hemolytic streptococci have not been studied.

Discussion. L forms were isolated rarely, from exceptional strains of a few species, before the effectiveness of penicillin to produce such forms was noticed. However, a great many strains could not be induced to produce L forms with the use of penicillin even though isolation of L colonies from other strains indicated that the particular species was capable of producing such forms. It is reasonable to expect that the technic described in this note—*i.e.,* the combined use of penicillin and the appropriate electrolyte concentration—will materially extend the number of positive results. If this expectation is correct this technic will also provide an opportunity for more conclusive study of the biological and pathological significance of the L forms.

The isolation of L forms from group A streptococci is especially interesting because of the obscure nature of the pathogenic role of the streptococcus in certain diseases.

At the present time no hypothesis can be proposed to explain the influence of salts on the production of L forms.

Summary. L type colonies have been isolated from group A streptococci by the combined effect of penicillin and concentrations of various salts considerably higher than usually incorporated in bacteriological media. The appearance and morphology of these cultures correspond to those isolated from the alpha streptococci. There is reason to believe that the technic described will be helpful in the isolation of L forms of other bacterial species.

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Dietary Conditions Affecting Oxidation of Tyrosine by Rat Liver, with Particular Reference to Thiamine.* (21298)

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The oxidation of tyrosine by mammalian liver enzymes proceeds through several steps before the end-products of the reaction, fumaric and acetoacetic acids, are obtained (1-6). Considerable work has been carried out to determine what cofactors are required for the various steps(5-11). Pyridoxal phosphate, ascorbic acid, and glutathione have been shown to be involved in the oxidation of tyrosine. Williams and Sreenivasan(9-11) found that the dye, 2,6-dichlorophenolindophenol (DCPP) could stimulate this oxidation in addition to the stimulation afforded by glutathione and ascorbic acid. It was considered possible that the DCPP in low concentrations might act as a substitute for a labile cofactor of an unknown nature. Boiled extracts of the active enzyme preparation also produced a stimulation of tyrosine oxidation by dialyzed or aged enzyme preparations in addition to the stimulation produced by glutathione and ascorbic acid.

Upon examination of the possible scheme for tyrosine oxidation, it becomes evident that the oxidation of 2,5-dihydroxyphenylpyruvic acid to homogentisic acid involves an oxidative decarboxylation. In view of the well known participation of thiamine-containing coenzymes in oxidative decarboxylation reactions, it was of interest to investigate the oxidation of tyrosine by enzyme preparations from livers of thiamine deficient rats as compared to those from normal control animals. If thiamine was active in promoting oxidation of tyrosine, its possible identity with the labile cofactor of the DCPP-substituting factor suggested by Williams and Sreenivasan(9-11) might be established.

Experimental. Since thiamine is active in the form of thiamine pyrophosphate (TPP) or, in certain systems, as lipothiamide pyrophosphate (LTPP)†(12), these substances were added to enzyme preparations obtained from normal and thiamine deficient rats. Dialyzed enzyme preparations, both from normal and thiamine deficient animals, have also been employed. A combination of a thiamine deficiency and dialysis of the enzyme preparations would be expected to remove thiamine-containing cofactors more completely than either treatment alone. Male rats of the Sprague-Dawley strain weight 90-110 g were divided into two groups. One group was fed a complete purified ration, and the other the same ration with thiamine omitted. The rations consisted of 20% vitamin-free casein,

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salts IV(13), 5% corn oil, 2% vitamin mix (14) (either complete or without thiamine), and 69% sucrose. The animals received food and water *ad libitum* and given weekly oral supplements of haliver oil fortified with vit E and K. After about 4 weeks on the respective diets, the animals receiving no thiamine had decreased in body weight by 20-30 g, while animals receiving complete ration had gained 150-175 g. Some deaths had occurred in the deficient group by this time. Animals from each group were sacrificed, the livers removed, and the enzyme prepared as previously described(9). Warburg flasks were prepared according to procedures already outlined(9). The various cofactors were added to the main compartment of the flasks, as indicated in the accompanying tables, at the following levels: ascorbic acid, 1000 γ; glutathione, 3120 γ; DCPP, 50 γ; TPP, 50 γ; and LTPP, 10 γ. The flasks were incubated in a Warburg bath at 37°C for 10 minutes before addition of the side arm contents. The stopcocks were then closed, and oxygen uptake recorded for one hour. Nitrogen of the enzyme preparations was determined using a semi-micro Kjeldahl procedure.

Results. The oxygen uptake data for liver enzymes prepared from normal and thiamine-deficient animals are presented in Table I. Contrary to all expectations it can be seen that, in the absence of any added cofactors, the tyrosine oxidase activity was considerably higher in those preparations which were obtained from thiamine deficient rats than from rats fed the complete ration. Addition of ascorbic acid and glutathione stimulated the activity in both normal and deficient preparations, although the degree of stimulation was significantly greater in the latter. Addition of the dye, DCPP, to these two preparations further increased oxidation of the substrate. The slight apparent stimulation by TPP in the presence of ascorbic acid and glutathione is not considered significant in view of the spread of the individual values which were averaged to obtain the figure in the Table. LTPP added in the presence of ascorbic acid and glutathione produced no alteration in effect when compared to that obtained when ascorbic acid and glutathione were added. Therefore, it appears that thiamine, when incorporated either as TPP or LTPP does not produce *in vitro* stimulation of the oxidation of tyrosine by either normal or thiamine-deficient enzyme preparations of rat liver under the conditions employed in these studies. When the activities were calculated on an enzyme nitrogen basis, essentially the same picture was obtained as when the results were expressed in terms of fresh liver.

TABLE I. Comparison of Liver Tyrosine Oxidase Preparations from Normal and Thiamine-Deficient Rats.

Additions	Normal activity*	Deficient activity*
None	30	48
AA,† GSH	177	229
" " , DCPP	211	505
" " , TPP	187	263
" " , DCPP, TPP	255	489
" " , LTPP	174	260
" " , DCPP, LTPP	254	514

* The data for addition of LTPP or TPP are averages of 3-4 experiments. Otherwise 8-9 determinations made. Values are expressed as μlO_2/flask for 60 min.

† AA, ascorbic acid; GSH, glutathione; DCPP, 2,6-dichlorophenolindophenol; TPP, thiamine pyrophosphate, LTPP, lipothiamidepyrophosphate.

TABLE II. Comparison of Liver Tyrosine Oxidase Preparations after Dialysis from Normal and Thiamine-Deficient Rats.

Additions	Normal activity*	Deficient activity*
None	0	11
AA, GSH	0	112
" " , DCPP	0	226
" " , TPP	0	162
" " " , DCPP	0	228
" " , LTPP	0	115
" " " , DCPP	0	236

* These data are averages of 6-7 experiments and expressed as μlO_2/mg nitrogen/hr.

Effect of dialysis of the normal and thiamine deficient preparations. Since it was possible that, even in the deficient preparations, sufficient thiamine might be present to mask the effect of added TPP or LTPP, dialysis of the homogenates was employed to remove cofactors as completely as possible. The enzyme was prepared as previously described(9) from both normal and deficient animals. The crude enzyme was then dialyzed against frequent changes of distilled water at 5° C for 7-9

TABLE III. Effect of *In Vivo* Thiamine Supplements on Tyrosine Oxidase Preparations from Normal and Thiamine-Deficient Animals.

	Additions	Normal activity*	Deficient activity*
A	None	21	80
	AA, GSH	150	278
B	None	28	29
	AA, GSH	167	168

* Part A is avg of 4-5 experiments. These animals were injected with 2 mg of thiamine 24 hr before use. Part B is the avg of 3 experiments. These animals were fed a complete ration 12-13 days before use. The values are expressed as $\mu l O_2$/mg N/hr.

days before enzymatic activity was measured. The results are presented in Table II. When no cofactor was added, oxygen uptake was small for the thiamine deficient group. Addition of the cofactors listed in the Table, however, increased the activity of the enzyme from the thiamine deficient animals 10-20 fold. In no case was any activity obtained under similar conditions for the enzyme preparations from normal control animals. The slight apparent stimulation by TPP in the presence of ascorbic acid and glutathione for the thiamine deficient group is not considered significant because of overlapping of values from this group and the control. Similarly, addition of LTPP to flasks containing ascorbic acid and glutathione produced no stimulation of oxidation of the substrate.

Effect of thiamine supplementation to deficient and normal animals. Since the activity of the tyrosine oxidase system was so greatly increased in liver preparations from thiamine deficient animals, the question whether the increase was a direct or an indirect consequence of the vitamin deficiency arose. In an effort to gain information on this, both thiamine deficient and control animals were injected intraperitoneally with 2 mg of thiamine. The day following the injections, the enzyme was prepared from livers and the tyrosine oxidase activity measured in the usual manner. Other thiamine deficient animals were transferred from the deficient to the complete diet, and after 12-13 days the livers were assayed for tyrosine oxidase activity. The results of these experiments are presented in Table III. Part A of the Table contains data for animals which were analyzed one day after injection of thiamine, and Part B contains results for those deficient animals which were resupplemented with a complete diet for 12-13 days. From Part A it can be seen that, when thiamine was injected into deficient animals, the tyrosine oxidase activity was still considerably higher in deficient than in normal animals. However, when a complete ration was fed to a group of thiamine-deficient animals for a period of about two weeks before analysis, the differences between deficient and control groups disappeared.

Effect of inanition on tyrosine oxidase activity. Since one of the more prominent features of a thiamine deficiency is anorexia, it was possible that the increase in tyrosine oxidase during a thiamine deficiency might have been due to a decrease in food intake. To study this possibility, the effects of inanition on liver tyrosine oxidase activity were investigated. Male rats of the Sprague-Dawley strain weighing 110-140 g were used. They were placed in cages with water but received no food for 4-7 days. At this time livers were removed and assayed for tyrosine oxidase activity. The results of these experiments, presented in Table IV, indicate that the system from starved animals could be stimulated by DCPP in the presence of ascorbic acid and glutathione to produce a greater oxidation of tyrosine than the normal preparation. This observation was also true of the system from thiamine-deficient animals. However, the stimulation by ascorbic acid and glutathione was not as marked in the preparations from starved animals as in those from thiamine-deficient animals.

Discussion. Increased oxidation of tyrosine by enzyme preparations from livers of thiamine-deficient rats or from starved rats as compared to the normal controls, is an unex-

TABLE IV. Comparison of Liver Tyrosine Oxidase Preparations from Normal and Starved Animals.*

System additions	Starved	Normal
None	31	35
AA, GSH	131	214
" " , DCPP	351	254

* These data are averages of 6-8 experiments and expressed as $\mu l O_2$/mg N/hr.

pected observation that is very difficult to explain. In many cases of nutritional insufficiencies, decreases in activity of many enzymes have been observed. Thus, inanition lowers the activity of liver xanthine oxidase(15) while feeding of a diet low in protein decreases the activity of xanthine oxidase(16), choline oxidase(17), and succinic oxidase(18). The activity of xanthine oxidase and succinic oxidase has also been decreased by feeding diets lacking in methionine(19). Therefore, the fact that a thiamine-deficiency increases the oxidation of tyrosine by liver preparations and that this effect can be partially duplicated by the effects of simple inanition is difficult to fit with what is known concerning interrelationships of nutritional deficiencies and tissue enzyme activities.

One possibly similar situation is that of the effect of a folic acid deficiency on liver xanthine oxidase activity. Several workers(20-23) have observed that in animals with a folic acid deficiency liver xanthine oxidase is considerably higher than in animals receiving a complete diet.

The failure to demonstrate a requirement for thiamine for the oxidative decarboxylation of tyrosine is quite unusual in view of the fact that similar reactions involving α-ketoglutarate and pyruvate have a definite need for the vitamin(22,23).

Summary. 1. Oxidation of tyrosine by the tyrosine oxidase system from livers of thiamine-deficient animals is markedly higher than that by preparations from normal animals. 2. After dialysis the activity of the normal preparation cannot be restored by the addition of ascorbic acid, glutathione, or 2,6-dichlorophenolindophenol (DCPP). The thiamine-deficient preparations are still very active under these conditions, however. 3. Injection of thiamine into normal and deficient animals one day before assay of the livers for tyrosine oxidase has little effect on the high activity of the thiamine-deficient preparations. Feeding a complete ration for 2 weeks to thiamine-deficient animals, however, decreases the activity to the level present in the normal preparations. 4. Inanition produces an increase in tyrosine oxidase activity by liver enzymes. This may be a partial explanation for the fact that thiamine-deficient rats (which are anorexic) possess a higher activity of this enzyme. The reason for the increase under either of these conditions, however, cannot be explained at present.

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Red Cell Life Span in Acclimatization to Altitude. (21299)

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It is well known that individuals going from sea level to higher altitude develop a secondary polycythemia, the level to which the total red cell volume rises being a function of the altitude(1,2). Two mechanisms can be postulated for the development of this polycythemia, the first is an increase in the rate of formation of red cells, and the second an increase in the life span of the red blood cell or possibly a combination of both. Earlier studies using radioactive iron demonstrated conclusively that there is an increase in the rate of production of red cells when individuals go from sea level to 14,900 feet(2). However, these studies did not exclude the possibility that some alteration in the red cell life span occurred so that the total red cell volume would increase both as a result of increased production and an increased life span of the red cell. In the rat, no change in red cell life could be demonstrated during the period of acclimatization to a simulated altitude of 15,000 feet(3). Studies were undertaken using Carbon-14 labelled glycine of the life span of the red blood cell of individuals going from sea level to 14,900 feet.

Methods. Six individuals normally living at sea level were taken to 14,900 feet, 4 hours being required to reach altitude, and then 10 hours after arrival at 14,900 feet, given 100 μc of glycine 2-C-14 intravenously for determination of the life span of the red blood cell according to the method of Shemin and Rittenberg(4). Blood samples were drawn at frequent intervals and the hemoglobin extracted by modification of the method of Drabkin (5,6). The hemoglobin was converted to CO_2 by the method of Van Slyke and Folch(7), and the radioactivity determined in a 100 cc ionization chamber at atmospheric pressure using a vibrating reed electrometer and recording potentiometer to measure the rate of voltage change in the ionization chamber(8).

TABLE I. Hematological Data at Sea Level and after 10 Days at 14900 Feet, in 6 Subjects.

		Hematological data (sea level)			Hematological data after 10 days at 14900 feet		
	Age	R.B.C.	Hgb.	Hct.	R.B.C.	Hgb.	Hct.
		M			M		
S.P.	22	5.30	16.5	48.0	6.10	18.5	54.6
J.R.	21	5.17	15.7	48.0	6.00	18.5	55.4
O.M.	20	5.33	16.5	49.0	6.32	19.5	59.1
A.C.	20	4.73	14.7	45.0	5.46	17.1	52.0
A.M.	22	5.22	16.4	50.4	6.20	19.3	57.4
L.S.	20	4.86	15.1	46.9	6.33	18.2	56.9

Results. Table I shows the pertinent hematological data in these individuals. The graph depicting the change in C-14 concentration in the hemoglobin of these individuals is shown in Fig. 1. This figure shows that the life span of the red blood cell varies from 111 to 121 days in these individuals and is normal.

Discussion. The observation of an increased red blood cell count, hemoglobin and hematocrit and an increased total red cell volume in the polycythemia secondary to low oxygen tension is thus shown to be exclusively

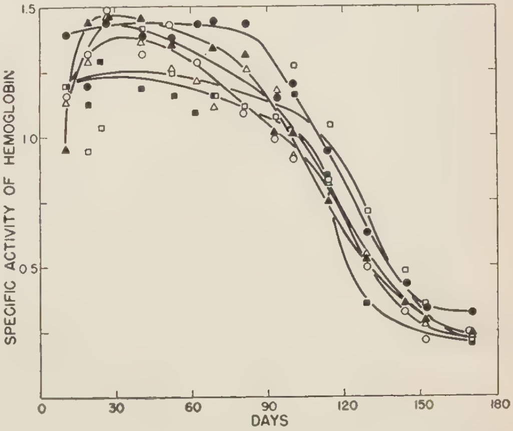

FIG. 1. Specific activity (in dis/min./mg $BaCO_3$) of hemoglobin.

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the result of an increase in the rate of production and not to be due to any alteration in the life span of the red blood cell.

Summary. Six individuals taken from sea level to 14,900 feet showed a normal red cell life span during the period of acclimatization; therefore, the polycythemia secondary to altitude is due entirely to an increase in the rate of red cell formation.

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Distribution of Glycoproteins in Normal Human Plasma.* (21300)

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(Introduced by Charles M. Carpenter.)

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The glycoproteins† of plasma are a heterogeneous group of conjugated proteins consisting of peptides in firm union with polysaccharides. Their occurrence, chemistry, significance, and methods for determination have been reviewed by Winzler(1). Due to the complex fractionation procedures required for the isolation of individual glycoproteins (2-4), the most generally employed method for the estimation of the protein-bound carbohydrate of plasma is the determination of total plasma glycoprotein. Although such determinations have yielded valuable data on the levels of plasma glycoproteins in many physiologic and pathologic conditions, they provide no information on the distribution of the protein-bound carbohydrate among the protein fractions present in blood plasma.

In the present investigation data obtained from the analyses of fractions isolated by a low temperature-low salt-ethanol procedure (5) from normal human plasma are correlated with the results of the more widely employed total plasma glycoprotein and seromucoid determinations.

Materials and methods. Plasma. Seventeen ml of blood were obtained by venipuncture from 10 male and 10 female donors in apparent good health and varying in age from 21 to 45 years. The blood was immediately added to 3 ml of A.C.D. solution(5). Following separation by centrifugation from the blood cells, the A.C.D. plasma was stored at −20°C until the fractionations and the chemical determinations could be conducted. *Fractionation.* Five ml aliquots of A.C.D. plasma were fractionated by the low temperature-low salt-ethanol procedure of Lever and associates (5). The separations were carried out in a −5°C bath containing 50% by volume of ethanol and equipped with an effective stirrer. Precipitates were recovered by centrifugation at −5°C. *Chemical analyses.* Fractions I + III, II, and VI were dissolved and made up to 10 ml and Fraction IV + V was dissolved and made up to 25 ml with the same reagents employed by Lever *et al.*(5). The protein-bound carbohydrate (hexose) content of the plasma fractions was determined as follows. One-ml

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† Terminology employed is in accord with the recent suggestions of Winzler(1). The term "total plasma glycoprotein" has replaced "total plasma polysaccharide" and "seromucoid" has been substituted for "mucoprotein."

TABLE I. Distribution of Protein-Bound Carbohydrate and Protein in Normal Human Plasma. Twenty samples in each determination.

	Protein-bound carbohydrate			Protein			Protein-bound carbohydrate ÷ protein × 100	
Component	Mean* (mg %)	CV†	% of total	Mean* (g %)	CV†	% of total	Mean* (%)	CV†
Total	109 ± 2.1	.086	100	7.4 ± .12	.072	100	1.5 ± .03	.093
Fraction I + III	38 ± 1.2	.141	34.9	1.3 ± .04	.138	17.6	2.9 ± .07	.108
" II	13 ± .7	.241	11.9	.9 ± .04	.199	12.2	1.4 ± .04	.128
" IV + V	41 ± 1.2	.131	37.6	4.9 ± .06	.055	66.2	.8 ± .03	.225
" VI	13 ± .5	.172	11.9	.2 ± .02	.447	2.7	6.5 ± .82	.538
Sum of primary fractions	105 ± 2.0		96.3	7.3 ± .10		98.7		
Albumin	12 ± .2‡	.075	11.0	4.0 ± .07	.078	54.0	.3§	
Fraction IV‖	29 ± .4	.062	26.6	.9 ± .06	.298	12.2	3.2 ± .22	.307
Seromucoid	10 ± .4	.179	9.2					

* Including stand. error of mean. † Coefficient of variation. ‡ Value calculated from albumin concentration and % of carbohydrate in human serum albumin (Cutter). § Value obtained by analysis of human serum albumin (salt-poor), Cutter Laboratories, Berkeley, Calif. ‖ Fraction IV values represent difference between values for Fraction IV + V and albumin.

aliquots of the protein solutions were added dropwise to 10 ml absolute ethanol at room temperature. After standing 10 minutes, the precipitate was centrifuged at 1800 rpm for 10 minutes. The ethanol was decanted and the precipitate drained by inverting the tube. One ml of distilled water was added and the hexose content determined by an orcinol reaction(6). The protein content of the isolated fractions was estimated in one ml aliquots by the biuret method of Weichselbaum(7). Albumin was determined in Fraction IV + V by the hematin-binding procedure of Rosenfeld and Surgenor(8). Total plasma glycoprotein, seromucoid, and total plasma protein were determined by methods previously reported(6). The mean, standard error of the mean, and coefficient of variation were determined by standard statistical methods(9).

Results. The summary of the results of the chemical analyses is presented in Table I.

Distribution of protein-bound carbohydrate. The greatest concentration of protein-bound carbohydrate occurred in Fraction IV + V (α-globulin + albumin). Seventy-one percent of the value for the combined fraction was attributable to Fraction IV. Albumin with the lowest carbohydrate content of any of the determined fractions, nevertheless, accounted for a substantial percentage of the total plasma glycoprotein due to its high concentration in plasma.

Although Fraction II (γ-globulin) has been studied extensively in relation to its antibody content and physicochemical properties, little attention has been directed to the polysaccharide moiety of the fraction. The results of the current study indicate that carbohydrate forms an integral part of the γ-globulin molecule. The hexose value for the fraction, 13 mg %, represented 11.9% of the total plasma glycoprotein.

Protein-bound carbohydrate values for Fractions II, V, and VI were similar, notwithstanding the large differences in the protein content of the fractions.

The polysaccharide level of the perchloric acid-soluble, phosphotungstic acid-insoluble seromucoid fraction was lower than the corresponding values of the fractions isolated by ethanol precipitation. The major component of Fraction VI and seromucoid is orosomucoid, a glycoprotein with a very low isoelectric point, pH 1.8, and characterized by a high degree of solubility(10).

The 4 primary fractions accounted for 96.3% of the total plasma glycoprotein. The difference is attributable to losses common to all fractionation procedures. Variability as measured by the coefficient of variation was most pronounced in Fraction II.

Distribution of protein. Fraction IV + V accounted for 66.2% of the total protein content of plasma. The concentration of albumin represented 54% of the total plasma protein. The ratio was in good agreement with plasma albumin values obtained by electrophoretic analyses(11,12). The calculated ratio for Fraction IV (α-globulin) in plasma was lower than corresponding values based on electrophoretic measurements(11,12). It is to be noted, however, that α-globulin is also a constituent of Fractions I + III and VI(5). The percentage of γ-globulin (Fraction II) was intermediate between 2 reported values obtained by electrophoresis(11,12). The sum of the protein concentrations of the 4 isolated fractions represented 98.7% of the total plasma protein. Individual values for Fraction VI were subject to the greatest degree of variability. The magnitude of the variation is due in part to the technical difficulties encountered in the determination of very low concentrations of protein by a biuret method.

Protein-bound carbohydrate as per cent of protein. Two of the 4 primary fractions (I + III and VI) were observed to have carbohydrate/protein ratios greater than the value obtained for whole plasma. Fraction IV + V with the lowest percentage of glycoprotein, however, accounted for two-thirds of the protein content and three-eighths of the protein-bound carbohydrate content of plasma. The protein-bound carbohydrate of Fraction VI represented 6.5% of the fraction, presumably due to the presence of orosomucoid, with a hexose content of 17%(4,10). The high hexose content of Fraction I + III, 2.9% was somewhat surprising in view of the low values obtained by similar determinations on β-globulin and fibrinogen from pooled, normal plasma(13). The possibility exists, however, that the relatively high value of the fraction is due to the presence of α-globulin, which represents approximately 17% of the protein content(5).

The fractionation procedure of Lever *et al.* (5) has certain limitations with respect to its employment in the study of plasma glycoproteins. The most important are the marked heterogeneity of Fraction I + III (α-globulin + β-globulin + fibrinogen) and the separation of α-globulin and albumin into the same Fraction IV + V. Albumin and α-globulin have been shown to decrease and increase, respectively, in a large number of pathologic and experimental conditions(14). The latter disadvantage has been obviated to a certain extent, as demonstrated in the current study, by the development of improved methods (8,15) for the determination of albumin in Fraction IV + V.

Advantages of the separation procedure include the isolation of fractions of known composition in an undenatured state, permitting the determination of their chemical, physical, and physiologic properties.

Summary. Five-ml samples of normal, human plasma have been separated by a low temperature-low salt-ethanol procedure into 4 primary fractions, I + III, II, IV + V, and VI. The concentrations of protein-bound carbohydrate (hexose) and of protein in whole plasma and the 4 primary fractions and the protein-bound carbohydrate content of seromucoid were determined. Fraction IV + V, of the primary fractions, contained the greatest amount of protein-bound carbohydrate and of protein. The percentage of protein-bound carbohydrate with respect to protein was highest in Fraction VI.

The assistance of Mrs. Gertrude Weinfeld in collecting the blood specimens is gratefully acknowledged.

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Effect of Caloric Restriction on Cholesterol Atherogenesis in the Rabbit. (21301)

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The effect of caloric restriction on the genesis of atherosclerosis has received very limited attention. Studies of human obesity(1-3) and surveys of the nutritional and health status in various countries(4,a,b) have resulted in a general impression that low caloric intake exerts a protective action in any atherogenic situation. Scanty information is available concerning the influence of caloric restriction on the development of experimental atherosclerosis(5,6). The present study was undertaken to furnish more detailed findings regarding the effects of severe caloric restriction in rabbits fed cholesterol.*

Our experimental results are significant because they 1) illustrate the fallacy of regarding severe caloric restriction as the opposite of obesity and 2) point out the necessity for paying careful attention to the diet and weight of rabbits subjected to experimental procedures or unusual diets designed to elucidate factors involved in atherogenesis.

Material and methods. Male, adult Swiss rabbits weighing between 3.5 and 5.5 kg were kept in the laboratory for several weeks prior to the experiments and the average daily food intake necessary to maintain weight equilibrium was estimated. A standard food mixture† was used which has the following content of nutrients, minerals and vitamins: fiber 15%, fat 3%, protein 17.50%, calcium 1.66%, P .37%, I .0033%, salt 1.00%; pantothenic acid 13-14 mg/lb., niacin 17.5 mg/lb., thiamin 1.28 mg/lb., riboflavin 3.40 mg/lb., biotin 0.16 mg/lb., vit. C .81 mg/lb., vit. D 450 USP units/lb., vit. E 30 mg/lb., choline 640 mg/lb. This diet was supplemented with 10-20 g/day raw carrots. Two series of experiments were carried out differing in 1) duration, 2) degree of caloric restriction, 3) dose of cholesterol, and 4) mode of cholesterol administration. The control groups in both experiments were fed *ad libitum;* the average consumption approximated 130 g/day of the standard ration. There were 18 animals in Exp. A and 22 in B. Half of this number were on restricted food intake, the rest served as control. *Duration.* Exp. A lasted 12 weeks, Exp. B 8 weeks. *Degree of caloric restriction.* The underfed group in Exp. A received 50 g/day, Exp. B 30 g/day of rabbit pellets plus 20 g raw carrot. This constituted approximately ½ and ⅓ of average control ration. *Dose of cholesterol.* 1 g/day in Exp. A and 2 g/day in Exp. B. Thus the absolute cholesterol content in the groups on adequate and on restricted calories was the same, but the percentile content varied, being considerably higher in the restricted diets. *Mode of Administration.* Measured amounts of crystalline cholesterol were mixed thoroughly with shredded raw carrots without adding any oil. In Exp. A the rabbits consumed the carrot-cholesterol mixture at the same time as their pellets. In Exp. B regular feeding was delayed until 4 to 5 hours after completion of cholesterol ingestion. The uneaten

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* While the manuscript was being prepared for publication, an article appeared by McMillan, Whiteside, and Duff (*J. Exp. Med.*, 1954, v99, 261) dealing with the same experimental problem. It is of interest that the findings and conclusions are virtually in agreement with ours.

† B-B Laboratory Rabbit Diet (Maritime Milling Co., Inc., Buffalo, N. Y.)

TABLE I. Average and Range of Values for Total Serum Cholesterol, Phospholipids, Fatty Acids and Lipoproteins in Rabbits Fed Cholesterol on Full and Restricted Rations. Ranges are in parentheses.

Experiment		A		B	
Diet group:		Full	Restricted ½	Full	Restricted ⅔
Dose of cholesterol:		1 g/day	1 g/day	2 g/day	2 g/day
Duration of exp.:		12 wk	12 wk	8 wk	8 wk
Avg wt, kg	I.*	4.3	4.54	3.71	3.85
	F.*	4.28	3.55	3.91	2.85
Avg wt change, kg		–.02	–.99	+.20	–1.00
Cholesterol, mg %	I.	29.7 (20.4–39.2)	31.0 (17.4–38.2)	50.9 (26.9–65.8)	43.0 (30.9–45.7)
	F.	236.0 (121–682)	943.0 (755–1.670)	330.0 (64–432)	786.0 (565–1012)
Phospholipids, mg %	I.	2.9 (2.4–3.7)	3.0 (2.3–3.4)	3.84 (2.2–5.7)	3.48 (2.6–5.6)
	F.	6.7 (5.3–15.2)	15.3 (9.5 25.4)	9.24 (6.0–18.0)	15.28 (13.1–18.0)
Fatty acids, mg %	I.	106.0 (79–128.0)	114.0 (82–162)	147.0 (82–220)	128.0 (81–220)
	F.	213.0 (141–590)	480.0 (412–852)	295.0 (164–491)	454.0 (338–685)
Lipoproteins, mg %					
S_f 0– 11	I.	—	—	—	—
	F.	—	—	215 (65–310)	205 (80–530)
11– 21	I.	19 (11– 36)	20 (10– 29)	—	—
	F.	159 (62–427)	561 (215–1030)	350 (93–780)	493 (340–680)
21– 35	I.	14 (6– 29)	16 (9– 21)	—	—
	F.	122 (30–440)	632 (355–1200)	278 (20–665)	509 (308–863)
35-100	I.	16 (4– 32)	27 (5– 46)	—	—
	F.	74 (29–243)	499 (333– 800)	278 (12–520)	482 (250–760)
100–400	I.	12 (2– 31)	20 (5– 41)	—	—
	F.	36 (12–130)	324 (76– 650)	207 (5–470)	246 (85–600)

* I. = Initial; F. = Final.

cholesterol was carefully gathered and weighed at the end of ingestion period. The average unit uneaten was 0.1 g with a variation of .05 to 0.2 g/day and no significant difference between control and underfed or between individual rabbits could be found. In both experiments the following biochemical tests were carried out: 1. Total blood cholesterol, using method of Abell, Levy, Brodie and Kendall (7); 2. Blood fatty acids, using method of Stern and Shapiro(8); 3. Lipid phosphorous using method of Youngburg and Youngburg (9); 4. Blood lipoproteins using method of Gofman and associates(10).[‡] Each determination was run in duplicates and agreement within 3% was required. In Exp. A these tests were made at beginning and after 5, 10 and 12 weeks; the lipoproteins were not determined at the 5 week interval. In Exp. B lipoproteins were determined only once, at the end of experiment, while the other tests were done at beginning and after 4 and 8 weeks. Blood samples were taken by heart puncture at beginning and end of experiments and by withdrawal from earveins at intervals.

Animals were weighed weekly. At end of experiments all animals were sacrificed and their aortas examined for degree of atherosclerosis. In Exp. A only a gross examination was done, in Exp. B gross inspection and microscopic examination were performed. A grading system from 1+ to 4+ was employed where 1+ indicated minimal lesions and 4+ extensive lesions with plaque formation involving entire length of vessel. In the present report only the range of values and their averages at beginning and end of experiments are given; individual findings will be reported elsewhere.

Results. Since both experiments gave rather similar results, they are reported together. The data are presented in Table I.

Weight. Control groups in Exp. A and B showed little change in their weight. The underfed group of 9 rabbits in Exp. A lost in 12 weeks an average of 0.99 kg or approximately 20% of initial weight. Only 2 animals lost less than 10% and one more than 30%

[‡] The lipoprotein determinations were processed in the Viral and Rickettsial Research Laboratories, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y. We wish to express our gratitude to Dr. Raymond A. Brown for his cooperation.

(42%). The 11 animals in Exp. B, kept under caloric restriction for 8 weeks, lost an average of 1 kg, or approximately 26% of initial weight with a variation from 19% to 31.2%.

Biochemical findings. Blood cholesterol showed in both experiments, a rise in control group as in the underfed group. In both experiments, however, the rise was much more pronounced and significantly higher in the underfed groups than in adequately fed groups. The larger dose of cholesterol in Exp. B was reflected also in a higher degree of cholesterolemia in animals on full diet and those on subnutrition. The shorter duration of this experiment and the different intervals at which the tests were taken do not permit, however, an exact comparison.

Blood fatty acids. In both experiments there was a marked increase in fatty acids. Again average values of underfed groups were markedly higher than those on full ration. The differences between these 2 groups were found to be statistically significant.

Lipid Phosphorous. There was a steady rise of lipid phosphorous in control as well as underfed groups. Again the values were continuously higher in subnutrition groups.

Lipo-proteins. Serial observations are available only for Exp. A where the S_f fractions 11/21, 21/35, 35/100, and 100/400 were determined at beginning and after 10 and 12 weeks. All fractions showed a gradual increase. In the full nutrition group the increases were relatively small and significant only in S_f 11/21, and 21/35. In the underfed group the differences were significant in all fractions, with the greatest increase in S_f 11/21 and S_f 21/35. In Exp. B, lipoproteins were determined at the end of the observation period only. Here the 0/11 fraction was also estimated. If the initial values of Exp. A can be used as a baseline, then statistically significant increases were observed in both control and underfed groups, with the latter showing still higher values than the former. Differences between both groups were not significant in S_f 0/11 and 100/400, but had statistical significance in S_f 11/21, 21/35 and 35/100.

Vascular changes. Gross inspection of the aortas after termination of experiments showed arteriosclerotic changes in both groups. Although advanced and extended lesions were found in animals on full rations and on restricted rations, the impression was that these were more pronounced in underfed animals. The anatomical differences were, however, less marked than the biochemical changes. In the underfed group of Exp. B, only one aorta had minimal lesions, 3 showed maximal lesions, and the changes in the remaining 7 were 2+ and 3+, while in the group on full nutrition, 5 aortas were practically free of degenerative changes, while 1 had maximal changes and the 5 remaining showed moderate lesions (2+ to 3+). The gross findings were confirmed by microscopic examination. The degree of severity of lesions, and the difference between control and underfed groups, might find special significance in view of the relatively short duration of observation (8 weeks). It is likely that a higher degree of alteration would have developed in the control groups also, had the experiment been permitted to run for 12 weeks or longer.

Discussion. It is evident from the results that cholesterol feeding caused significant biochemical changes and atherogenesis both in animals on full nutrition as well as those on caloric restriction, the changes being even more severe in underfed group of rabbits. It will be noted that in both feeding groups, the rise of the cholesterol level is not as high as that observed by other investigators. This difference is probably explained by the fact that cholesterol was fed in our experiments without the admixture of other lipids. This fact may also be of interest in view of the observed increase of fatty acid levels in both groups of animals, the control as well as underfed groups.

From these experiments it appears that in the presence of high cholesterol intake, caloric restriction not only does not give any protection against degenerative vascular disease, but seems to enhance it. Similar observations were made by Rodbard, Bolene and Katz(6) in cholesterol atheromatosis of the chick, while Firstbrook(5) reported in his rabbit experiments a parallel between degree of cholesterol atherogenesis and caloric intake. In man, Walker(2) found that weight reduction without dietary cholesterol restriction is associated with significant reduction of the lipoprotein fractions, particularly the larger aggregates, and a fall in total blood cholesterol level. Whether this difference in results represents a species difference or is due to different amounts of cholesterol ingested will have to be explained. The dietary cholesterol in our experiment was proportionally far higher than that in Walker's observations.

Summary. 1. Rabbits on severe caloric restriction were fed cholesterol and showed significantly increased blood cholesterol, fatty acids, lipoproteins and phospholipids. The degree of these biochemical changes and of the atherosclerosis was greater than that of a control group fed similar amounts of cholesterol in addition to a normal diet. 2. Therefore, severe caloric restriction does not protect, but enhances cholesterol induced atherosclerosis in rabbits. This effect of weight loss should be taken into consideration in experiments on atherosclerosis.

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Effect of Chronic Administration of Glucagon to Rats and Rabbits.* (21302)

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Glucagon, the hyperglycemic-glycogenolytic factor of the pancreas, causes a transient increase in blood sugar when it is administered parenterally. Until Staub(1) developed methods suitable for the preparation of highly purified material of standard potency, it was not possible to study the effect of chronic administration of glucagon. This study was undertaken in an attempt to investigate the question: Will glucagon produce diabetes? Daily administration of large doses of glucagon to rats and rabbits for 6 months did not produce a diabetic state, but it did exert an interesting effect on liver glycogen stores.

Method. Rats and rabbits were injected with glucagon daily for 6 months. Thirty-eight rats, housed in individual cages in air-conditioned animal quarters, received a commercial rat food and water *ad libitum.* Thirty of the rats were injected intraperitoneally each morning with 1 mg of a highly purified, non-crystalline, preparation of glucagon (lot 208-108B-284) in a volume of 0.25 ml. The hyperglycemic potency of this lot is such that 1 mg corresponds to approximately 0.14 mg of crystalline glucagon. This preparation contains less than 0.005 unit of insulin per mg of glucagon. The remaining 8 rats were maintained as control animals and received no treatment. Sixteen rabbits housed in air-conditioned quarters were fed rabbit pellets and water *ad libitum.* Six of these animals were injected intravenously twice a day (7:30 a.m. and 4:00 p.m.) 7 days a week with 1 mg of

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*I am indebted to Dr. Paul N. Harris for performing the autopsies and carrying out the studies on pathology of the animals. My thanks are due Messers. Thomas Lenahan, Zane Frank, and Darrel Caldwell for their invaluable assistance in these experiments.

TABLE I. Effect of Chronic Administration of Glucagon, 1.0 mg per Day Intraperitoneally for 6 Months on Liver Glycogen of Rats.

	Control	Glucagon treated	P
No. of rats	8	29	
Liver wt, g	14.94± .97*	16.41 ± .49*	>.05
Liver glycogen concentration, mg/g	49.01±2.87	66.66 ±3.41	.04
Total liver glycogen, g	.74± .077	1.059± .070	.03

* Mean ± stand. error of the mean.

glucagon and the other 10 received only the morning injection. All the rats and rabbits were weighed once a week. Blood sugar values were determined on the rabbits once a week and on the rats once a month. Nonfasting blood samples were taken before the morning glucagon injection, and blood sugar was determined by the Hagedorn-Jensen method(2). At the end of 6 months all the animals still alive were killed by the administration of secobarbital sodium.† The abdomen was opened and duplicate samples of liver were quickly removed, weighed, and placed in tubes containing hot 30% KOH solution for glycogen determination by the method of Good, Kramer, and Somogyi(3). The rest of the liver was removed and weighed. The animals were examined for gross pathological changes, and material was taken for histological examination of liver, kidney, adrenal, pancreas, heart, lung, thyroid, and pituitary.

Results. 1. *Rats.* During the 6-month test one rat died with severe pulmonary congestion in the 19th week of treatment with glucagon. The other 37 survived the entire test period. There were no significant differences in weight gains between treated and control groups. The average monthly blood sugar values for all the treated animals ranged from 97 to 120 mg %, and for the control animals from 84 to 137 mg %. There was no detectable difference between the 2 groups. At the conclusion of the experiment all the rats were healthy and all contained large fat pads within the abdominal cavity. Neither gross nor histological examination revealed any organic lesions. Table I presents a comparison of liver weights, liver glycogen concentration, and total liver glycogen of the treated and control animals. Glucagon caused an increase in the concentration of glycogen in the livers of the treated animals without a significant increase in liver weight.

2. *Rabbits.* None of the 6 rabbits injected with glucagon twice daily survived a full 6 months. Two died during the 6th week and one each during the 7th, 8th, 9th, and 10th week of treatment. The blood sugar values for all these rabbits were within normal range throughout the experimental period. No changes in the organs of these animals were observed either grossly or microscopically. The cause of death was ascertained in only one of this group, that one animal showing marked pulmonary edema. Of the 10 rabbits injected once daily, 3 died during the 6 months, one each during the 5th, 7th, and 8th weeks of treatment. At autopsy all 3 animals showed evidence of marked pulmonary infection. The 7 animals that lived to the end of the 6-month period gained weight at a normal rate except for the 3- or 4-week period during which all of the deaths occurred. At that time a severe respiratory infection was present in the rabbit colony. The average weight of the group at the start of the experiment was 3.236 kg and at the conclusion 4.204 kg. Post-mortem examination at the end of 6 months showed the animals to be fat and healthy. Gross examination of the aortae did not reveal any evidence of thickening or atherosclerotic plaques. All tissues studied were normal.

In Table II the liver glycogen values of the

TABLE II. Effect of Daily Intravenous Administration of Glucagon on the Liver Glycogen in Rabbits.

	Control	Glucagon treated	P
No. of rabbits	5	7	
Body wt, kg	5.02	4.204	
Liver wt, g	90.70± 5.91*	110.91±7.18*	>.05
Liver glycogen concentration, mg/g	64.42±13.23	96.09±6.98	.04
Total liver glycogen, g	6.19± 1.69	10.94±1.55	.04

* Mean ± stand. error of the mean.

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† 'Seconal Sodium' (Secobarbital Sodium, Lilly).

glucagon-treated rabbits are compared with those of a group of untreated rabbits. The untreated animals had been in the laboratory quarters only a week before being killed. The concentration of glycogen in the livers of the glucagon-treated rabbits, calculated as mg/g of liver, was one and one-half times that of the untreated animals. Although comparisons between groups of animals not treated in parallel series cannot be conclusive, the results in rabbits combined with those in rats indicate that one effect of chronic administration of glucagon is to increase the concentration of glycogen in liver tissue. Further experiments in rats to be reported confirm this effect.

Discussion. Daily administration of glucagon to rats and rabbits for a period of 6 months did not alter the rate of growth of the animals, nor cause pathological changes in any of the organs or tissues studied. There was no permanent or lasting alteration of blood sugar and the animals did not have any symptoms of diabetes. The animals that died during the 6-month period, succumbed to infectious processes and not to drug action.

The only effect that could be ascribed to glucagon administration was an increase in the concentration of glycogen in the livers of both rats and rabbits. This surprising finding is the opposite of that expected on the basis of the known action of glucagon. Sutherland and Cori(5) demonstrated that glucagon causes an increase in active phosphorylase in liver and thereby increases glycogenolysis. This mechanism explains the rise in blood sugar and decrease in liver glycogen found after single injections of glucagon(6).

If active phosphorylase can also catalyze the glucose-l-phosphate → glycogen reaction, it is possible that chronic administration of large doses of glucagon will favor glycogen formation.

It is unlikely that this increase in liver glycogen is a secondary effect mediated via adrenal cortical hormones, since administration of ACTH increases liver glycogen stores by an increase in liver weight without altering the concentration of glycogen in the liver tissue (4). After glucagon administration there was an increase in liver glycogen with no significant increase in liver weight.

Summary. Daily administration of large doses of glucagon to rats and rabbits for 6 months did not produce any toxic reactions. The rate of weight gain of treated animals was the same as that of control animals. Diabetes was not produced and there was no evidence of chronic hyperglycemia. The liver glycogen concentration of the treated animals was significantly greater than that of the untreated animals.

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Studies on Production of Antipoliomyelitis Serum in Rabbits.* (21303)

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Many investigators(1) have attempted without success to produce poliomyelitis in the rabbit. Furthermore, completely negative results have also been reported(2-4) of attempts to produce neutralizing antibodies in rabbits inoculated repeatedly with virus infected monkey spinal cord suspensions given by various routes of inoculation, including the subcutaneous, intradermal, intraperitoneal and intramuscular. On the other hand, antipoliomyelitis sera have been produced successfully in horses, sheep(5,6), and mice(7).

To our knowledge there have been no reports of the development of neutralizing antibodies in rabbits inoculated with poliomyelitis virus grown in tissue culture. The present report deals with the successful production of type specific neutralizing antibodies by rabbits inoculated with the 3 types of poliomyelitis virus propagated in tissue culture. Both active and ultraviolet irradiated virus antigens were employed.

Material and method. The active and irradiated suspensions used in these studies were prepared from poliomyelitis virus propagated in monkey kidney tissue culture. Each strain of virus was grown individually, titrated, and its identity was checked with type specific antiserum. Table I contains titration data relative to the pools of virus types used for rabbit immunization. Ultraviolet irradiation was carried out with each virus type pool individually as described previously(8). Virus pool titrations and serum neutralization tests were done in test tube cultures of monkey kidney tissue fragments. In all antibody titrations pre and post vaccination serum samples were routinely tested simultaneously. The virus serum mixtures were seeded into tissue culture tubes and contained 100 50% tissue culture infecting doses of virus ($TCID_{50}$) in final mixture with each dilution of serum. Serial dilutions of serum beginning with undiluted (1/1), 1/5, 1/10, etc. were mixed with equal volumes of virus suspension and inoculated into 2 or 3 tubes per serum level. Titers are expressed as the 50% end point and represent the dilution of serum that was added to the virus before inoculation of the tissue culture tubes. New Zealand reds and albino rabbits weighing 3 to 5 lbs were used for immunization. All rabbits were bled by cardiac puncture prior to immunization and at subsequent intervals following immunization. Since we were uncertain of antibody responses, rabbits were immunized repeatedly by the intravenous or intramuscular routes in accordance with the following trial dosage schedules: *Schedule A. Injections.* 5 cc intramuscularly plus 5 cc or 10 cc intravenously on day 1 followed by 10 cc intravenously on days 4, 8, 11, 15, 18 and 22. 1 cc or 10 cc intravenously on day 60. *Bleedings.* Preimmunization specimen day 1, day 28 (R specimen), day 48 (F specimen), day 67 (K specimen). *Schedule B.* 5 cc intramuscularly plus 5 cc intravenously day 1. 10 cc intravenously day 31. 1 cc intravenously day 61. *Bleedings.* Preimmunization specimen day 1, day 38 (R specimen), day 65 (F specimen), day 85 (K specimen). *Schedule C.* 5 cc intra-

TABLE I. Titrations of Pools of Poliomyelitis Virus Used in Production of Rabbit Type Specific Antisera.*

Virus strain	Type	Pool No.	Titer, negative log of 50% end point
Mahoney	I	TC 85	4.0
Brunhilde	I	TC102	3.5
MEF1	II	TC 75	3.0
MEF1	II	TC 88	3.0
Saukett	III	TC 86	5.0

* Titrated in monkey kidney fragment tissue culture with 0.1 cc inoculum per dilution.

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* Supported in part by a grant from Armour Laboratories.

† Deceased.

TABLE II. Antibody Response of 15 Rabbits Inoculated with Active or Irradiated Type 1 (Mahoney or Brunhilde Strain) Poliomyelitis Virus by Intravenous or Intramuscular Route.

			Active virus			Irradiated virus		
Rabbit No.	Type	Dosage schedule	R sample	F sample	K sample	R sample	F sample	K sample
1	1 (Mahoney)	A	>1/320	1/160	1/320	—	—	—
2	”	A	”	†		—	—	—
5 S*	”	A	1/320	1/5260	>1/21, 140	—	—	—
6 S*	”	A	”	”	” ”	—	—	—
6	”	A	—	—	—	1/80	1/20	1/320
8	”	A	—	—	—	1/80	1/20	1/640
7 S*	”	A	—	—	—	1/12	<1/5	<1/12
8 S*	”	A	—	—	—	1/320	1/80	>1/320
10 S	1 (Brunhilde)	A	>1/21, 140	1/320	>1/21, 140	—	—	—
11 S	”	A	—	—	—	1/108	<1/80	1/108
12 S	”	A	—	—	—	1/108	<1/80	1/320
4	1 (Mahoney)	B	1/160	1/80	1/80	—	—	—
5	”	B	1/640	1/640	<1/320	—	—	—
9	”	B	—	—	—	1/10	†	
10	”	B	—	—	—	1/40	—	—
13 S	”	C	1/1280	>1/320	>1/1280	—	—	—
14 S	”	C	1/108	1/80	1/320	—	—	—
15 S	”	C	—	—	—	1/80	<1/80	>1/320
16 S	”	C	—	—	—	<1/5	—	—

* Initial and final inoculation 10 cc.
† Rabbit died.

muscularly days 1, 8, 15, and 46. *Bleedings.* Preimmunization specimen day 1, day 22 (R specimen), day 45 (F specimen), day 53 (K specimen).

Results. Immunization with active and irradiated type 1 poliomyelitis virus given intravenously. Comparative results obtained in the immunization of 19 rabbits with Type 1 (Mahoney or Brunhilde strains) with ultraviolet inactivated or active virus, according to dosage Schedule A, are outlined in Table II. None of the rabbits had detectable antibodies for the 3 types of poliomyelitis virus in the prevaccination serum specimens, and, therefore, these negative findings are omitted from all tables. In postvaccination specimens only type specific antibodies were produced and no instances of cross neutralization were observed. There was an excellent specific homotypic antibody response to active Mahoney or Brunhilde Type 1 poliomyelitis virus (Table II). It is also apparent that there is considerable variation in the antibody response of the individual rabbit. The irradiated Type 1 Mahoney virus also produced a significant rise in homotypic antibody titer but not to the same degree as the active virus. Too few rabbits were inoculated with the Brunhilde Type 1 virus to draw definite conclusions, but the superiority of active virus is demonstrated in the only rabbit which survived of the two inoculated. The inactivated virus produced a homotypic antibody response which was definitely inferior to that of the active virus. Rabbits immunized with only 3 injections intravenously of Type 1 Mahoney virus, either active or irradiated, failed to develop antibody levels comparable to those elicited in the rabbits that had received 7 inoculations in the previous experiment (Table II). This result is not unexpected because of the much smaller quantity of antigen injected.

Immunization with Type 1 virus intramuscularly. Four rabbits were inoculated intramuscularly with active or irradiated Type 1 Mahoney strain according to dosage Schedule C. Results obtained are included in Table II. Although only 3 rabbits were available for study of all post-vaccination blood specimens, it is noteworthy that the results approximate those obtained by repeated intravenous inoculations (Table II).

Immunization with Type 2 virus inoculated intravenously. Four rabbits were immunized with one combined intramuscular and intravenous inoculation followed by 6 intravenous inoculations according to dosage Schedule A.

TABLE III. Antibody Response of Rabbits Inoculated Intravenously with Active or Irradiated Type 2 (MEF1 Strain) and Type 3 (Saukett Strain) Poliomyelitis Virus (Dosage Schedule A).

Rabbit No.	Type	R serum sample
	Active virus	
21	2	1/320
22	2	1/80
25	3	1/160
26	3	1/40
	Irradiated virus	
23	2	1/160
24	2	1/160
27	3	1/40
32	3	<1/20

Type 2 MEF1 strain active and irradiated virus suspensions were used. Results obtained are given in Table III. No antibodies were detected in the prevaccination serums and there was a homotypic antibody response to Type 2 virus. The results obtained with the irradiated virus were nearly comparable to those of the active virus.

Immunization with Type 3 virus inoculated intravenously. Four rabbits were immunized with 7 intravenous inoculations according to dosage Schedule A with Type 3 (Saukett) active and irradiated virus. Table III outlines the results obtained. No antibodies were detected in prevaccination serums and there was a homotypic antibody response to Type 3 virus. Although the results obtained represent the inoculation of only 4 rabbits, the active virus appears to be somewhat superior in immunogenicity to the irradiated virus.

Immunization with trivalent irradiated vaccine intravenously. As a matter of interest, 8 rabbits were immunized with 7 intravenous inoculations of trivalent irradiated tissue culture vaccine according to dosage Schedule A. The vaccine was composed of equal parts of Type 1 (Brunhilde strain), Type 2 (MEF1 strain) and Type 3 (Saukett strain). Results obtained with the irradiated vaccine are shown in Table IV. It is evident that the trivalent irradiated vaccine stimulated a significant antibody response to the 3 types of poliomyelitis virus in most of the rabbits that were inoculated.

Discussion. The present studies reveal a significant antibody response of the rabbit to intravenous or intramuscular inoculations of the three types of poliomyelitis virus grown in tissue culture. In no instance were antibodies detected in the preimmunization serums of the inoculated rabbits. Both active and ultraviolet irradiated virus were effective in stimulating antibody formation in the rabbit, although the former was definitely superior in most experiments. The antibody response obtained in rabbits inoculated with active or irradiated tissue culture virus was surprisingly good in view of the fact that these early experimental preparations used were not optimal and represent initial efforts at both virus growth and irradiation. Antibody response was proportional to the amount of antigen and number of inoculations given as shown in Table V. Three inoculations of 5cc intramuscularly at weekly intervals were approximately equivalent to seven 10cc inoculations given twice weekly by the intravenous route. The antibody response to each virus type was homotypic. There were inadequate numbers of albino and pigmented rabbits to afford a comparison of antibody responses in these two varieties.

TABLE IV. Antibody Response of Rabbits Inoculated Intravenously with Trivalent Irradiated Vaccine (Dosage Schedule A).

Rabbit No.	R serum sample type 1	2	3
47	1/8	1/64	1/32
48	1/8	1/16	1/1
49	1/8	>1/32	1/16
50	>1/32	>1/32	1/16
51	1/8	1/1	1/1
52	1/1	1/8	1/4
53	1/4	>1/32	<1/1
54	1/8	1/64	1/1

TABLE V. Antibody Response of Rabbits Inoculated Intramuscularly with Varying Amounts of Active Type 1 (Brunhilde Strain) Virus (Dosage Schedule C).

Rabbit No.	Amount inj., cc	R serum sample Type 1
17 S	.05	>1:1280
18 S	.05	1:320
20 S	.5	1:1280
21 S	.5	1:80
22 S	.5	1:80
23 S	5.0	>1:5260
24 S	5.0	1:1280
25 S	5.0	1:5260

Although an occasional rabbit died of intercurrent infection, there was no "clinical" evidence that any of the 3 types of tissue culture virus was pathogenic for the rabbit, thus confirming the results of earlier workers(1). The variability in antibody responses of individual rabbits emphasizes the need of using adequate numbers in each test—possibly 5 animals per specimen. The response of the rabbit to the trivalent irradiated vaccine suggests the possible use of a rabbit test for assay of the immunogenic potency of different batches of vaccine for the selection of more antigenic strains of virus for vaccine production, and for determining the antigenicity of vaccines inactivated by various chemical or physical methods. Finally, storage tests of different types of poliomyelitis vaccine may be carried out.

Summary. Type specific neutralizing antibodies were produced in rabbits inoculated with the 3 types of poliomyelitis virus grown in tissue culture. Rabbits were successfully immunized by repeated intravenous or intramuscular inoculations of active or ultraviolet irradiated virus suspensions. The possibilities of a rabbit test for the assay of poliomyelitis vaccine are discussed.

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Endogenous Infection in Mice Irradiated with Fast Neutrons or Gamma Rays.* (21304)

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The development of generalized infection has been shown to play a significant role in the death of mice exposed to moderate doses (450-600 r) of total body x-irradiation in a single exposure(1,2). Such infections, which were always caused by bacteria of enteric origin, were demonstrated by blood and spleen cultures of mice killed at daily intervals after x-irradiation, and in a more recent study, by serial blood cultures(3). Bacteremia occurred most frequently during the second week following x-irradiation. In mice exposed to an equivalent dose of fast neutrons, death has been found to occur much earlier—between the 4th and 8th day(4), *i.e.*, before bacteremia becomes an important factor as a cause of death following x-irradiation. Field studies utilizing nuclear test devices in 1951 and 1952 had demonstrated this difference in mortality time between neutron- and γ-irradiated mice(5). This shorter survival time of neutron-irradiated mice raised the question whether generalized infection has time to develop and contribute to the lethal effect as it does in x-irradiated mice. The following study was undertaken to determine the incidence of bacteremia in mice irradiated with fast neutrons and, for comparison, mice irradiated with gamma rays from Cobalt 60.

Methods and materials. Female CF No. 1 mice, 5-6 weeks old, were shipped[†] to the laboratory by air express and placed in isolation for 3 weeks. They were maintained on a diet of Rockland mouse pellets and water

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[*] Part of this investigation was carried out under contract between the U. S. Atomic Energy Commission and the University of Chicago.

[†] From Carworth Farms, New City, N. Y.

ad libitum, and weighed 20-25 g when irradiated. Eight mice were housed together in a plastic box (11 x 7 x 5½ inches) containing wood shavings(6). *Methods of irradiation.* The mice were irradiated at the Argonne National Laboratory in the gamma-neutron radiation chamber described by Vogel *et al.* (7). During exposure the animals were kept at approximately 75°F and 50% relative humidity. Fast neutrons were produced through fission, by allowing neutrons from the thermal column of the Argonne CP-3′ reactor to impinge on uranium. Appropriate shielding devices of boron carbide prevented slow neutrons from reaching the animal exposure position. The gamma contamination of the fast neutrons was approximately 7%(8). Pure gamma radiation was obtained from 18 sources of Cobalt 60 located at the opposite end of the chamber from the uranium. Mice were irradiated in a lucite chamber, 16 in. x 16 in. x 2 in., containing 66 exposure cells, each of which could hold one animal. The intensity at each animal position was determined and isodose groups were established for both neutron and gamma radiations(9). *Dosimetry.* During irradiation, Victoreen condenser r-chambers were inserted into standard positions in the dosimetry columns of the lucite animal cage(7). With the aid of Dr. H. H. Rossi of Columbia University, a Victoreen chamber was calibrated with a tissue equivalent chamber of 10.1% hydrogen composition so that doses in the fast neutron field could be expressed in terms of roentgens-equivalent-physical (rep) (8). This calibration has recently been confirmed by Mills and Blosser of Oak Ridge National Laboratory using a Hurst proportional counter for neutron dosimetry(10). One of the Victoreen 25 r thimble chambers with 4 mm plastic cap has also been calibrated, for gamma radiation from Co^{60} in terms of International Roentgens, by the National Bureau of Standards. *Dose range.* 188 mice were irradiated with fast neutrons in 4 experiments. The dose rate was 2.5-3.0 rep/min. and the time of exposure varied from 75 to 90 minutes. Previous work had established the LD_{50}—30 days for this strain to be 210 rep of fast neutrons(11). The dose range used (216-265 rep) was thus in the upper lethal range, between approximately the LD_{60} and the LD_{98}—30 days. 111 mice were exposed to Co^{60} γ rays in 2 experiments. The duration of the first exposure was 75 minutes (dose rate: 12-12.5 r/min.). The second irradiation lasted approximately 45 minutes, since the dose rate was increased to 20.4-21.7 r/min. The LD_{50} (single 90-minute exposure) for this strain to Co^{60} γ rays, is approximately 920 r (11). Thus the exposures used in these experiments (900-942 r) were in the mid-lethal range (LD_{40-60}—30 days), and were comparable to, but lower than, the fast neutron exposures. *Animal transportation.* Immediately after irradiation the mice were transported 25 miles to the University of Chicago in an air conditioned truck(12). Thereafter they were housed in a mouse room[‡] maintained at 75°F and 52% relative humidity. Their plastic cages were chemically sterilized and changed twice a week. Sterilized water bottles were refilled daily and replaced weekly; food was provided in mangers. *Autopsy and culture methods.* On the days indicated below, mice were killed with ether, the abdomen opened aseptically, the spleen removed, cut in several places, and dropped into a tube of brain-heart infusion broth. The chest cavity was opened, the heart seared with a hot spatula and as much blood as possible drawn into a capillary pipette. One drop was streaked on each of 3 agar plates—blood, eosin-methylene-blue and Salmonella-Shigella agars. The remainder of the blood was cultured in brain-heart infusion broth. A record was kept of the number of colonies that developed on the agar plates. As soon as growth appeared in the broths, subcultures onto differential media were made. If no growth was visible by the 3rd day, they were subcultured onto blood agar before they were discarded. All microorganisms recovered from these cultures were identified by their morphology, gram reaction and characteristic appearance on appropriate media. These included the ones already mentioned as well as triple sugar-iron agar, urea broth and the usual carbohydrate broths. Anaerobic cultures were not made because anaerobes were recovered from only 0.3% of blood and/or spleen cultures of x-irradiated mice(1). *Neutron-irradiated mice.* From the 3rd day post-irradiation through the 11th, 5 mice a day (each from a different cage) were killed for culture of heart's blood and spleen. This procedure was followed for each of the 4 neutron experiments until 129 of the 188 exposed mice had been cultured, a total of approximately 20 per day during the period of greatest mortality—3rd-8th days post-irradiation (Table III). Forty-six of the mice that died were also autopsied for inspection of the viscera and for heart's blood culture. *Gamma-irradiated mice.* From the 6th through the 15th days post-irradiation, 5 to 10 mice (from different cages) were killed each day for culture of heart's blood and spleen. *Control cultures on normal mice.* Blood and spleen cultures were also made on 36 unirradiated control mice which were transported to and from the reactor together with the irradiated animals. These control mice were sacrificed and cultured at daily intervals using the methods described. All of their cultures remained sterile.

[‡] The authors wish to acknowledge the various facilities that were made available to them in the Argonne Cancer Research Hospital.

Results. The neutron irradiated mice, despite their earlier deaths, showed conclusive evidence of the development of generalized infection. The mortality figures, represented by the line graphs in Fig. 1, were taken from data used to construct survival curves for CF No. 1 female mice, exposed to single 90-minute radiations of fast neutrons (948 mice) or to Co^{60} gamma rays (1154 mice)(11). Each daily mortality figure is the per cent of the total number which died in the 30-day post-irradiation period (447 deaths following fast neutrons; 579 after gamma rays). The peak of the daily mortality curve for fast neutrons occurred 4-6 days after irradiation, and for gamma rays 12-14 days post-irradiation. No significant difference in the mortality pattern was observed between the mice which died in these experiments and those presented in Fig. 1. The results of the heart's blood and spleen cultures are shown in the bar graphs as per cent of the mice killed and cultured each day which had positive cultures of heart's blood and spleen (stippled bars) or positive cultures of spleen only (striated bars). The per cent of mice with positive blood cultures would have been increased if the figures had included cultures of the mice which died. The results of these post mortem cultures have been omitted from the data illustrated in Fig. 1, but are shown in Table III.

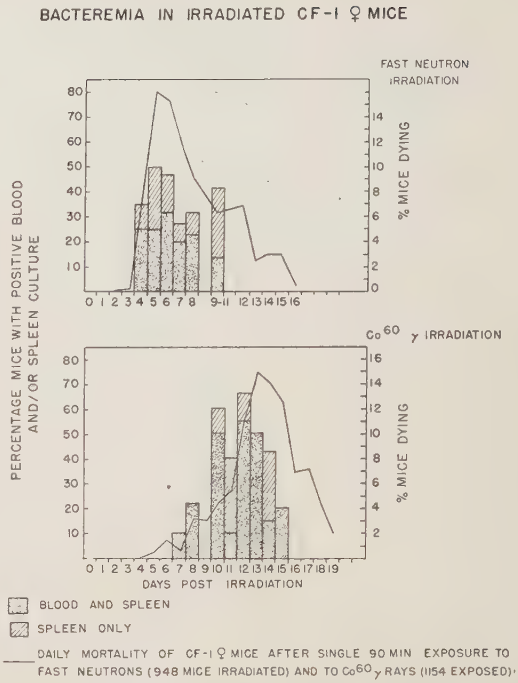

FIG. 1. Bacteremia and mortality in CF #1 mice irradiated with fast neutrons and gamma rays.

Neutron-irradiated mice. Beginning on the 4th day, heart's blood and/or spleen cultures were positive in ⅓ to ½ of the sacrificed mice. The onset of bacteremia, therefore, coincided with the steep rise in the mortality curve, and both maxima occurred on the 5th or 6th day. It is worth noting that on the 3rd day, when mortality was insignificant, cultures on all 20 of the mice were negative. Of the neutron-irradiated mice killed for bacteriological examination, 26 had positive cultures of blood and

TABLE I. Numbers of Bacteria Recovered from Blood Cultures.

Colonies/drop of blood	Neutron mice, %	Gamma mice, %
Innumerable	—	70
>50	—	15
25-50	4	10
1-25	46	—
Only broth positive	50	4

TABLE II. Classification of Microorganisms Recovered from Heart's Blood and Spleen.

	Neutron-irradiated mice, %	Co^{60} γ-irradiated mice, %
E. coli	55	18
Proteus	20	4
α-Streptococcus	11	71
Paracolobactrum	7	7
Alcaligenes	4	—
Gram neg. bacilli unclassified	3	—

spleen, 17 of spleen only. The numbers of colonies which developed from a capillary drop of blood are given in Table I, which shows that the severity of the bacteremia was less in the neutron- than in the gamma-irradiated mice. The microorganisms recovered from these cultures are listed in Table II according to their per cent distribution among the several genera or species. All of the microorganisms were members of the normal intestinal flora of these mice. The cultures obtained from the neutron-irradiated mice were, with few exceptions, pure cultures. One blood culture and 3 spleen cultures contained 2 kinds of microorganisms. As shown in Table III, the fraction of positive blood cultures obtained from the mice which died was nearly twice that obtained from those which were sacrificed for culture. The results on cultures of dead mice have been excluded from the data presented in the graph in order to eliminate the consideration of post mortem contamination.

Gross pathological findings. All mice were carefully autopsied, but no histopathological studies were made. The following changes were noted: Evidence of diarrhea; distention of small and large bowel with fluid after the 4th day post-irradiation; progressive decrease in size of the spleen to the 9th day; obvious anemia after the 6th day; rare petechial hemorrhages beneath the visceral peritoneum after the 6th day.

TABLE III. Positive Cultures on Sacrificed Mice and on Mice Which Died following Neutron Irradiation.

Post-irradiation day	No. positive cultures/No. mice cultured	
	Sacrificed mice	Dead mice
4	7/20	4/13
5	10/20	11/12
6	9/19	6/10
7	4/15	6/8
8	7/21	2/3
Totals	37/95 39%	29/46 63%

Gamma-irradiated mice. The highest incidence of positive cultures and of deaths did not occur until the latter part of the 2nd week following the gamma irradiation (Fig. 1). Since these results, as well as the severity of the bacteremia (Table I), duplicated so closely the earlier findings on x-irradiated mice (1,2), it seemed unnecessary to secure additional data. The principal difference between the results on the gamma-irradiated mice and those already published on x-irradiated mice was the recovery of alpha streptococci from a large proportion of blood cultures in the present series (Table II). Most of these streptococcal bacteremias occurred in one of the 2 groups of gamma-irradiated animals. We have previously observed differences, though usually less striking than this, in the percentage distribution of the several microorganisms recovered in blood cultures from different groups of mice. That was one of the reasons for repeating the earlier experiments on so many groups of animals.

Discussion. It is apparent from these experiments that mice irradiated with fast neutrons, despite their shorter survival time, developed generalized infection, demonstrated by the presence of bacteria in blood and/or spleen, as did mice exposed to gamma rays from Co^{60} or to X-rays(1,2). The data illustrated in Fig. 1 give the impression that the onset of bacteremia in the neutron-irradiated mice coincided in time with the steep rise in the mortality curve but did not precede it, as in the case of the gamma-irradiated mice. The reality of this apparent difference was questioned for the following reasons: (a) Although both radiations were administered in the upper lethal range, the neutron doses used (mean dose: 235 rep (LD$_{85}$—30 days)) were higher than were the gamma ray doses (mean dose: 935 r (LD$_{52}$—30 days)); (b) the vertical scale in Fig. 1, showing per cent positive cultures, was drawn arbitrarily at 5 times the scale representing per cent mortality of the mice; (c) the mortality data were represented as per cent decedent mice dying each day rather than as daily probability of death.

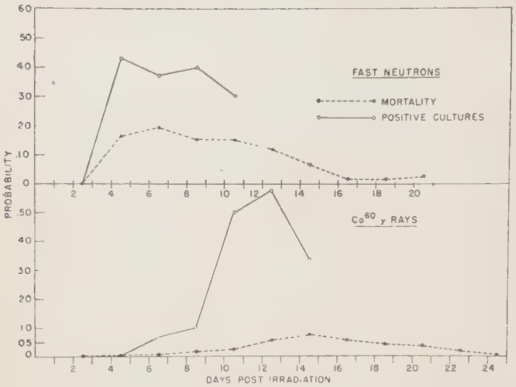

FIG. 2. Comparison of daily probabilities of death and of positive cultures of blood and/or spleen.

Therefore, a comparison was made (Fig. 2) between the daily probabilities of death and of positive cultures in the mice, following each ionizing radiation. By this means 2 rates could be directly compared. The daily probabilities, represented in this figure, have been normalized over 2-day periods. The mortality data have been carefully selected for the actual dose ranges of each radiation used. Since the dose was higher, the probability of death was higher in the neutron-irradiated than in the γ-irradiated mice. The peak of positive cultures preceded the peak of mortality in both cases. In other words, following either radiation, the maximum incidence of positive cultures occurred slightly before the maximum mortality. More frequent sampling between the 3rd and 5th days after neutron exposure, or serial blood cultures on individual mice, might have detected the onset of generalized infection before any deaths had occurred.

Bacteremias in the neutron-irradiated mice were less severe, as measured by colony counts, than bacteremias in the gamma-irradiated mice, or the x-irradiated mice previously reported. This difference is an interesting one and deserves further investigation. It suggests that among their injurious effects which lead to death, neutrons cause relatively less impairment of the mouse's resistance to infection than do gamma or X-rays.

There have been few previous studies on the role of infection as a factor in the mortality of mice after fast neutron irradiation. Lawrence and Tennant(13) exposed Swiss mice to 200 kv X-rays and to neutrons generated by a cyclotron (beryllium bombarded by 5 mev deuterons). All animals dying after irradiation were carefully examined post mortem, and bacteriological studies were included. The organisms isolated were found to be normal inhabitants of the gastrointestinal tract. They concluded that infection was not a consistent finding in mice dying within a few days after large doses of X-ray or neutrons, but that bacteremia was commonly found if mice were exposed to smaller doses of radiation which permitted them to live longer. They described numerous ulcers in the radiosensitive intestinal mucosa and focal necroses in the livers of animals dying 9-14 days after irradiation, all of which had positive blood cultures.

Silverman, Bond, Chin, and Greenman(14) have recently reported on the role of infection in the death of mice (CF No. 1 males) exposed to high doses of fast neutrons from the 60-inch cyclotron at Berkeley. The estimated dose was 9.5-10.6 x 10^{10} n/cm^2 measured by sulfur threshold detectors. It should be noted that this neutron dose probably exceeded 500 rep and might well be termed supra-lethal since, under our experimental conditions, 250 rep is the LD_{99} (20 days). It should be appreciated, however, that spectral differences existed between the 2 radiation sources employed. The biological significance of these differences, in terms of their effect on the ultimate biological response to measured rep doses, is not fully understood. These workers also found that bacteremia and mortality were correlated in time, and they recovered enteric bacteria in approximately the same proportion as reported here(15). Their results, however, differed from those herein reported in 2 respects: a higher incidence of mixed cultures, *i.e.*, containing 2 or more kinds of microorganisms, and an earlier onset of bacteremia and death (mean survival time 3-4 days). These differences can probably be attributed to the greater dose of neutrons used. None of the mice in their experiments survived more than 4 or 5 days.

It seems to be established, therefore, that generalized infection including invasion of the blood stream, occurs in mice following a single acute irradiation with fast neutrons. The cause-effect relationship, *i.e.*, the relative importance of infection in the death of neutron-irradiated mice, will be discussed in a later communication which will report the results of antibiotic therapy.

Summary. 1. Single, 90-minute irradiation with fast neutrons within the lethal range (175-250 rep) resulted in early deaths among CF No. 1 female mice with a significant peak in the mortality curve 4-8 days after exposure. Among 129 neutron-irradiated mice (216-265 rep: LD_{60-98}—30 days), sacrificed for bacteriological examination, positive cultures of heart's blood and/or spleen were first obtained on the 4th day, and coincided with the onset of mortality. Between the 4th and 8th days after exposure, approximately 40% of the sacrificed mice showed positive cultures. 2. After comparable irradiation with gamma rays (Co^{60}), from 750-1300 r, deaths occurred later, with a peak approximately 12-14 days post-exposure. Gamma-irradiated mice (900-940 r) were found to have positive blood and/or spleen cultures during the latter part of the second week. There seems to be a close correlation between bacteremia and time of death following these 2 ionizing radiations. 3. All the microorganisms recovered in cultures were found to be members of the normal enteric flora of the mouse. 4. The bacteremia, as measured by colony counts on heart's blood, was less severe in the neutron-irradiated than in the γ-irradiated mice.

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Measurement of Glomerular Filtration Rate in the Dog.*† (21305)

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Although it is well established that the clearances of inulin and exogenous creatinine in the dog are essentially identical, there is no direct evidence that either of these substances is excreted solely by glomerular filtration in the mammalian kidney. There is, however, direct histochemical evidence that the ferrocyanide ion is filtered at the glomerulus and is neither reabsorbed nor secreted by the tubular epithelium. This has been demonstrated in the rabbit(1,2), in the dog(3) and in the puppy(4). Van Slyke *et al.*(5) compared the renal clearances and extraction percentages of ferrocyanide, exogenous creatinine and inulin in the dog and concluded that they were excreted by the same mechanism. The wide spread of their data, however, and their use of the single intravenous injection technique, leave their conclusion open to criticism. Berliner *et al.*(6) reported that 109 clearance

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TABLE I. Clearance Ratios.*

Clearance ratio	No. of periods	Avg ratio	Range in ratios	Ratios between .95–1.05, %	Ratios between .90–1.10, %	Plasma conc., mg %
All C/I	87	.998	.845–1.17	71 = 82	85 = 97.5	C .38–11.1 I 9 –57
En C/I	55	.999	.91 –1.17	46 = 83.5	54 = 98.5	C .38– .915 I 11 –57
Ex C/I	32	.990	.845–1.06	25 = 78	31 = 97	C 1.4 –11.8 I 9 –48.5
All C/All F	92	1.008	.90 –1.15	62 = 67.5	90 = 98	C .38–11.8 F 2.1 –23
All C/Fh	67	1.006	.90 –1.10	49 = 73	67 =100	C .38–11.8 F 6.6 –23
All C/Fl	25	1.013	.90 –1.15	13 = 52	23 = 92.5	C .5 – .915 F 2.1 – 3.2
En C/All F	60	1.006	.90 –1.15	39 = 65	58 = 96.5	C .38– .915 F 2.1 –18.6
Ex C/All F	32	1.010	.92 –1.08	23 = 72	32 =100	C 1.4 –11.8 F 6.6 –23
All F/I	82	.987	.88 –1.11	58 = 70.5	77 = 94	F 2.1 –23 I 9 –57
Fh/I	61	.981	.88 –1.11	43 = 70.5	57 = 93.5	F 6.6 –23 I 9 –57
Fl/I	21	1.009	.88 –1.07	15 = 71.5	20 = 95.5	F 2.1 – 3.2 I 19 –45

* C = Creatinine; En = Endogenous; Ex = Exogenous. I = Inulin; F = Ferrocyanide; h = High conc.; l = Low conc.

comparisons of ferrocyanide and exogenous creatinine in the dog showed that the ferrocyanide clearance averaged 96.6% of the simultaneous creatinine clearance. A consistent difference of this magnitude may be due to some systematic error or could be due to a real difference in excretion mechanisms. It seemed desirable, therefore, to reinvestigate the renal excretion of ferrocyanide by the dog as well as the mechanism of excretion of endogenous creatinine.

Procedure. Nine trained unanesthetized female dogs were the subjects for 97 clearance comparisons in 21 experiments. Inulin, sodium ferrocyanide and creatinine, when it was given, were injected subcutaneously or given by constant intravenous infusion. Urine was collected through an inlying urethral catheter into a volumetric flask, and the bladder was washed with saline at the end of each period. An arterial blood sample was collected as nearly as possible at the midpoint of the period. *Chemical Techniques.* Inulin was determined in diluted urine and cadmium filtrates of serum by a modification of the resorcinol technique. Creatinine, both endogenous and exogenous, was determined by the technique of Hare(7). Ferrocyanide in concentrations greater than 5 mg% was determined by the method of Husson(8). A modification of this technique was devised for use at lower concentrations.

Results. The data obtained are summarized in Table I. In the 82 periods in which simultaneous clearances of inulin, ferrocyanide and creatinine were compared, all 3 agreed within 5% in 44 (53.5%), and within 10% in 76 (93%). The clearances ranged from 30-110 cc/min.

Discussion. Since on the average, over a wide range of plasma concentrations, no 2 clearances differ by as much as 2%, all the substances studied seem to be cleared at the same rate. Only 9 individual ratios, out of a total of 261, showed that any 2 clearances differed by more than 10%, and in nearly every case the serum concentration of one or both substances was changing during that period.

A systematic error in chemical technique will show up in the final average ratio of all the periods. It should be pointed out here that some serum samples have been encountered from which added ferrocyanide cannot be completely recovered either by the Husson technique or the modified procedure. Recovery is complete from most serum samples, but the possibility that there is some binding of ferrocyanide to serum protein *in vivo* has not been ruled out. However, the fact that the ferrocyanide clearance agrees with both inulin and creatinine clearance so closely at high and low plasma concentrations, indicates that the ferrocyanide determined in the serum must approximate very closely that which is filterable at the glomerulus.

Conclusion. Inulin, exogenous and endogenous creatinine, and ferrocyanide ion are all cleared at the same rate by the dog kidney, and probably exclusively by glomerular filtration.

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Vitamin B_{12} and Sub-Optimal Levels of Pantothenic Acid in Chick Nutrition.* (21306)

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The syndrome caused by the lack of pantothenic acid was reported by Norris and Ringrose(1) to be characterized by retarded and rough feather growth, definite, crusty, scab-like lesions in the corners of the mouth, and sometimes a cracking of the epithelium between the toes.

The pantothenic acid requirement for the chick has been placed at 5.5 mg/kg by Bauernfeind *et al.*(2) and at 14.3 mg/kg by Jukes(3). More recently, the National Research Council (4) recommended the use of 11.0 mg/kg of feed. The requirement of the chick for vit. B_{12} has been placed at 11.0 μg/kg by the National Research Council(4).

In view of the reports by Evans *et al.*(5) and Yacowitz *et al.*(6) concerning the effect of vit. B_{12} upon the pantothenic acid content of the liver, it appeared desirable to investigate the effect of vit. B_{12} upon the pantothenic acid metabolism when a sub-optimal amount (based on the National Research Council recommendations of 1950) of pantothenic acid was fed. In addition, the effect of pantothenic acid on B_{12} metabolism was investigated. This was done by making observations on growth, deficiency symptoms, and liver storage of B_{12} and pantothenic acid in 4-week old chicks.

Material and methods. Unsexed New Hampshire chicks were randomly distributed into 8 groups of 12 birds each. The birds were weighed, wing-banded, and placed in electrically heated batteries with raised wire floors. After the initial weighing, the chicks were weighed at weekly intervals during the 4 week experiment. The basal diet consisted of 25% purified soybean protein†, 67% glucose (cere-

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* Cerelose was supplied through the courtesy of the Corn Products Refining Co., Argo, Ill. Folic acid was supplied by Lederle Laboratories, Pearl River, N. Y.; biotin by Hoffmann-La Roche, Inc., Nutley, N. J.; and the rest of the B-vitamins by Merck and Co., Rahway, N. J. Methionine was obtained from Dow Chemical Co., Freeport, Texas.

† Drackett 220 obtained from the Drackett Co., Cincinnati, O.

TABLE I. Effect of Vitamin B_{12} and Pantothenic Acid on Growth, Pantothenic Acid Deficiency Symptoms, Mortality and B_{12} and Pantothenic Acid Content of Chick Livers.

Supplement to basal	Avg wt at 4 wk (g)	Severity of deficiency		% mortality	B_{12} content, mμg/g	PA content, μg/g
		Mouth*	Feet†			
PA‡ (1.66 mg/kg)	141.0	.88	.00	57.8	66.4	61.7
B_{12} (50 μg/kg)	100.2	1.17	.07	73.6	176.4	62.2
PA (1.66 mg/kg) + B_{12} (50 μg/kg)	130.9	1.17	.25	36.8	152.0	62.4
PA (" ") + B_{12} (100 ")	124.4	1.40	.40	47.5	302.8	65.7
PA (" ") + B_{12} (250 ")	109.0	1.58	.58	36.8	267.8	62.5
PA (3.32 ") + B_{12} (50 ")	204.8	.19	.00	15.8	201.9	62.4
PA (6.64 ") + B_{12} (50 ")	314.0	.00	.00	00.0	239.0	85.4
PA (9.96 ") + B_{12} (50 ")	315.9	.00	.00	15.8	215.5	102.4

* Dermatitis about mouth. † Cracking of epithelial tissue between toes. ‡ Pantothenic acid.

lose), 5% salts IV (Hegsted *et al.*, 7), and 3% refined soybean oil. Methionine and glycine were added to all diets at the levels of 7.5 and 4 g/kg, respectively. In addition, the following were added in the specified number of mg/kg of diet: thiamin hydrochloride, 4; riboflavin, 6; pyridoxine hydrochloride, 4; niacin, 100; folic acid, 2; biotin, 0.2; 2-methyl-1, 4-naphthoquinone, 0.5; and alpha-tocopheryl acetate, 3. Choline was added at the level of 2 g/kg. Vitamins A and D were added at the levels of 10000 I.U. and 2000 I.C.U./kg respectively. The plan of the experiment is shown in Table I. At the end of the 4 week experimental period, the chicks were sacrificed and the livers removed for vitamin determinations. A 2.5 g portion of liver was placed in a graduated cylinder and water added to 50 ml. This was then homogenized in a Waring blendor for 5 minutes. A 10 ml aliquot (500 mg of liver) was removed for vit. B_{12} determinations. A 2 ml aliquot (100 mg of liver) was removed for pantothenic acid determinations. Vit. B_{12} was liberated by incubating the homogenate at 37° C for 24 hours in the presence of pancreatin. The assay was carried out according to the procedure of Skeggs *et al.*(8) with *Lactobacillus leichmannii* 4797 as the test organism. Acid production at the end of 72 hours was used as the index of growth. Pantothenic acid was liberated by incubating for 3 hours at 37° C in the presence of a pH 8.3 buffer, intestinal phosphatase, and pigeon liver enzyme. Pantothenic acid analyses were carried out using *Lactobacillus arabinosus* 8014 according to the method of Skeggs and Wright(9). Acid production at the end of 72 hours was used as an index of growth. The birds were examined at the end of the experiment and scored for dermatitis about the mouth and for cracks in the epithelial tissue between the toes. In order to arrive at some means of expressing these results, the deficiency symptoms were calculated separately. The severity of the deficiency was scored as +, 2+, or 3+ with 3+ indicating the most severe. Each of the +'s was then counted as a unit. The total number of +'s for each symptom was then divided by the total number of birds in that group. The result obtained expresses the degree of severity of each symptom per bird in that group.

Results. Growth. The average weight of the chicks was decreased when B_{12} (50 μg/kg) was added to the low pantothenic acid (1.66 mg/kg) diet (Table I). A further reduction in growth occurred as the level of B_{12} was increased. The severity of deficiency symptoms also increased as the addition of B_{12} was increased (Table I).

The average weight of the birds was increased by increasing the level of pantothenic acid to 3.32 and 6.64 mg/kg respectively. The latter mentioned level was adequate for the prevention of the deficiency symptoms. The soybean protein used in this study supplied 10.5 mg of pantothenic acid/kg of diet (PA content supplied by Drackett Co.). This value plus the 6.64 mg of supplemental pantothenic acid, which was the lowest level that was effective in promoting adequate growth and preventing dermatitis, gives a figure of 17.14 mg/kg. This is only slightly higher than the level reported by Jukes(3).

B_{12} and pantothenic acid content of liver. The B_{12} content of liver was increased by ad-

dition of B_{12} to the diet. The addition of 50 μg of B_{12} to the basal diet containing 1.66 mg of added pantothenic acid per kg increased the B_{12} content from 66.4 to 152.0 mγ/g of liver. The B_{12} storage in the liver was further increased to 302.8 mγ/g by increasing the B_{12} content of the diet to 100 μg/kg. An additional increase in the B_{12} level of the diet did not further increase the B_{12} content of the liver above that obtained with 100 μg of B_{12}. These observations are in agreement with the earlier work of Couch *et al.* (10).

The addition of 1.66 mg of pantothenic acid per kg of diet to the basal diet containing 50 μg of B_{12} had no effect upon the B_{12} level in the liver. The B_{12} content of the liver was increased to 201.9 mγ/g when 3.32 mg of pantothenic acid was added to the diet, and to 239.0 mγ/g when 6.64 mg of pantothenic acid were added. No further effect on the B_{12} in the liver was produced by adding 9.96 mg pantothenic acid per kg of diet. This indicates that pantothenic acid may have had a sparing effect on vit. B_{12}.

The pantothenic acid content of the liver was increased by the addition of 6.64 mg or 9.96 mg of pantothenic acid per kg of diet. The addition of pantothenic acid at these levels increased the total pantothenic acid content of the liver to 85.4 and 102.4 μg/g of liver, respectively. These values are somewhat lower than that found in other experiments where a higher level of supplemental pantothenic acid was fed. All levels of pantothenic acid fed below the 6.64 mg/kg level had a pantothenic acid content in the liver ranging from 61.7 to 65.7 μg/g. The pantothenic acid content of the liver was not influenced by the addition of vit. B_{12} to the diet.

Summary. 1. Growth of birds fed a pantothenic acid-low diet was decreased by addition of vit. B_{12}. The degree of severity of pantothenic acid deficiency symptoms observed was increased by addition of vit. B_{12}. The lowest level of supplemental pantothenic acid that supported growth and prevented deficiency symptoms was 6.64 mg/kg of diet or 17.14 mg/kg when pantothenic acid contributed by the protein is added. When the supplemental pantothenic acid level was increased to 9.96 mg/kg, no additional effect was observed. 2. The B_{12} content of liver was increased by addition of that vitamin to the chick diet and this was proportional to the level of B_{12} added. Additional increase in B_{12} content of the liver was observed when the pantothenic acid level in the diet was increased. This indicates that pantothenic acid may have had a sparing effect on B_{12} when the pantothenic acid content of the diet was adequate. 3. The pantothenic acid content of the liver was influenced only when the level of supplemental pantothenic acid was raised to 6.64 mg/kg or a total of 17.14 mg/kg or higher.

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A Reversal of the Anuria Occurring During Diffusion Respiration. (21307)

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Although the term, "neurogenic anuria" is frequently used in clinical literature to describe the onset of acute renal dysfunction, it is generally difficult to produce experimentally such anurias in normotensive, non-traumatized, well hydrated animals. Draper and Whitehead have described a rapidly developing anuria, the onset of which roughly parallels the onset of respiratory arrest resulting from overdoses of injection anesthetics(1-3). This anuria could be largely reversed by the "pharmacological" denervation of the kidney, and hence appears to be neurogenic in nature. Although the mechanisms of such anuria may well lend insight into the general nature of acute renal dysfunction (of neurogenic and other origins) little is as yet known as to why it occurs. This lack of knowledge is largely due to the difficulty of studying renal function when urine formation is markedly suppressed. Work done by the authors has demonstrated that the anuria occurring during diffusion respiration can be reversed by the use of an osmotic diuretic. This work is presented in the succeeding paragraphs.

Methods and materials. Thirty adult mongrel dogs of both sexes and varying weights were anesthetized with 20 mg/kg of body weight of thiopental sodium. Oxygen was administered endotracheally throughout the experiment, and all animals were denitrogenated one hour prior to the onset of diffusion respiration. Respiratory excursions were recorded by means of a tambour-lever system. Carotid blood pressures were recorded kymographically by means of a mercury manometer. Two drop counting devices consisting of a secondary relay circuit activating a signal magnet when a drop of urine formed across a break in the primary circuit were employed for counting urine drops. Each ureter was cannulated individually. The animals were divided into 3 groups of 10 each. The first group of 10 animals received approximately 15 ml/kg of body weight of isotonic saline intravenously throughout the experiment. Isotonic saline was administered in a similar fashion to a second group, but was replaced with isotonic mannitol following 20 minutes of diffusion respiration. The third group of animals received isotonic mannitol throughout the experiment. Following a control period during which blood pressure, urine formation and respiratory activity were recorded, diffusion respiration was initiated by slowly administering a quantity of thiopental sodium just adequate to suspend respiration. Small doses of thiopental sodium were administered periodically to maintain the respiratory arrest.

Results. Following the onset of diffusion respiration, a profound oliguria or total anuria resulted in all animals receiving normal saline. A similar trend toward anuria resulted during diffusion respiration in the second group of

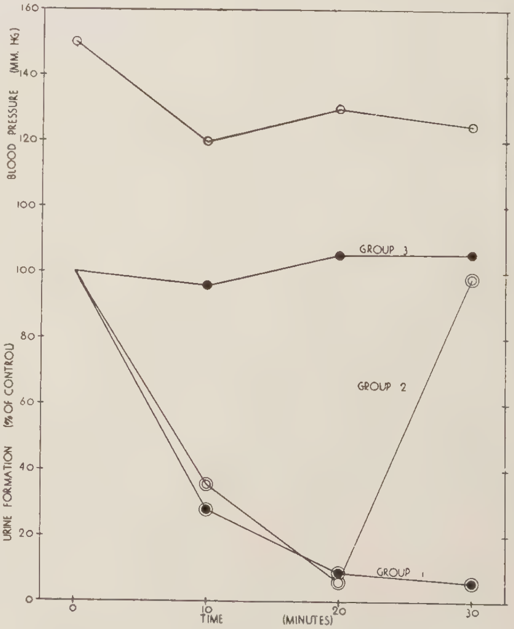

FIG. 1. Average response of urine formation to respiratory depression. Zero time represents onset of diffusion respiration. Group 1 received saline throughout experiment; group 2 received saline replaced by mannitol following 20 min. of respiratory arrest. Group 3 received mannitol throughout.

animals. This trend could be completely reversed in some animals and partially reversed in the remainder of the group by administration of mannitol. The group, receiving mannitol throughout the experiment (Group 3), maintained urine volumes, which in most instances closely approximated the control urine volumes throughout the period of diffusion respiration. Results of these experiments are shown in Fig. 1.

Discussion. One of the more recent and more promising methods for producing acute renal dysfunction in experimental animals is the technic described by Draper and Whitehead(1-3). However, little study has been given to the mechanisms involved in its development. The present work lends some insight into the development of this type of anuria, since the production of urine, even with a diuretic, would have been impossible if some glomerular filtration had not occurred. However, more important than this small insight into the mechanisms of this type of renal dysfunction is the fact that this anuria may prove a valuable tool for probing the role of neurogenic as well as other factors in acute renal dysfunction, and that the ability to reverse it with an osmotic diuretic may prove equally valuable as a means of facilitating such investigations.

Summary. Dogs, well hydrated with saline became anuric or oliguric when their respiration was suspended with overdoses of thiopental sodium. This anuria could either be reversed by administration of an isotonic mannitol solution, or did not occur when mannitol was administered throughout the experiment. The ability to reverse an experimental anuria with an osmotic diuretic presents a potentially valuable tool for the study of this type of renal dysfunction.

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"Collagenase" Activity of Cathepsins.* (21308)

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Collagen is a rather unique protein since it is not digested at neutral pH by any of the common proteinases. Its turnover rate in the normal adult mammalian organism is exceedingly slow(1) and little is known about the enzymes or mechanisms concerned with its synthesis or degradation. The only conclusively demonstrated collagenases have been described in culture filtrates of *Clostridium perfringens* and *C. histolyticum*(2). In the course of studies on collagenase activity, it was observed that the plant cathepsins, papain and ficin, and animal cathepsins were able to digest collagen by a mechanism differing from that of the bacterial collagenases, and involving a preliminary reversible alteration in the state of the substrate. It is the purpose of this report to describe these studies.

Methods. Collagenase activity was measured by the soluble nitrogen or soluble hydroxyproline peptides released from a weighed portion of purified Achilles tendon and is expressed in terms of percent digestion. Fifty mg of dried tendon was suspended in 4 ml of a buffer-enzyme mixture (3:1) and incubated for 3 hours at 37° C. After incubation, 5 ml of 10% NaCl was added and the mixture filtered. Kjeldahl analyses for nitrogen were performed on 1 ml aliquots of the filtrate. The percent digestion was calculated from the

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value obtained for soluble nitrogen, after correction for controls and reagents, and from the total nitrogen present in the collagen preparation. For hydroxyproline analyses, aliquots were hydrolyzed by 6 N HCl for 24 hours at 110°C, and the hydroxyproline determined (3). Purified bovine Achilles tendon was prepared by the method of Neuman and Tytell (4). Proteolysis of native and urea-denatured hemoglobin was determined by the method of Anson(5). 0.1 M buffers were prepared from the following acids: HCl, pH 1.5; H_3PO_4, pH 1.5 - 2.6; lactic acid, pH 3.0 - 4.5; acetic acid, pH 5 - 6; phosphate, pH 6 - 8; boric acid, pH 9 - 10. Commercially available preparations of papain, ficin, and crystalline trypsin were employed. Preparations of beef cathepsins were prepared according to the method of Tallan, Jones and Fruton(6).[‡] The animal and plant cathepsins were activated by 0.01 M or 0.005 M cysteine. *C. histolyticum* collagenase was obtained from 18-20 hour cultures of the organism, strain CHT.[§] The culture was filtered through a layer of Supercel, and the filtrate brought to 40% saturation with $(NH_4)_2SO_4$. The precipitate was redissolved in water, dialyzed and either used directly or after lyophilization.

Results. In Fig. 1 is shown the collagenase activity of papain, ficin, and the *C. histolyticum* collagenase preparation as a function of pH. The percent digestion is calculated from soluble nitrogen determinations. Hydroxyproline assays yielded identical results. No hydrolysis of collagen was evident with acid alone.

The activity of papain and ficin was optimal in the pH range of 2.0 to 4.5 and practically nonexistent near neutrality. By contrast, the bacterial collagenase had its optimum activity on collagen in the pH range from 6 to 8. The observations with papain and ficin were surprising since the pH optimum of ficin and papain against most protein substrates is near neutrality(7). An explanation for this phenomenon was suggested when the pH optimum of papain and ficin against native and urea-denatured hemoglobin was investigated (Fig. 2).

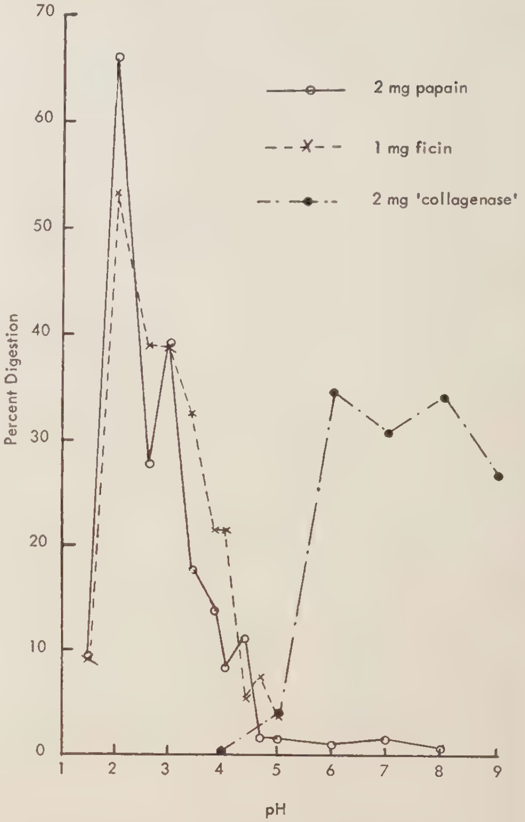

FIG. 1. pH optima of papain, ficin, and *C. histolyticum* collagenase on Achilles tendon.

It can be seen from the data in Fig. 2 that both papain and ficin optimally attacked urea-denatured hemoglobin at pH 7-8, but attacked native hemoglobin optimally at pH 4.0. The data suggested that the enhancement of enzymatic attack on native hemoglobin at pH 4 was due to the state of the substrate rather than a characteristic of the enzyme, since native hemoglobin is no longer in the "native" state at pH 4, undergoing an irreversible acid denaturation. Additional evidence for this view was obtained by investigating the pH optimum of crystalline trypsin on denatured and native hemoglobin. As suspected, the optimum observed with urea-denatured hemoglobin was in the region of neutrality, and with native hemoglobin at pH 4. Very little proteolysis by trypsin was evident with native hemoglobin at neutrality.

[‡] Our cathepsin B + C corresponds to their solution 4, and our cathepsin C to their solution 5.

[§] Culture medium contained 20 g/L BBL Trypticase, 0.48 g/L KH_2PO_4, and 2.28 g/L Na_2HPO_4.

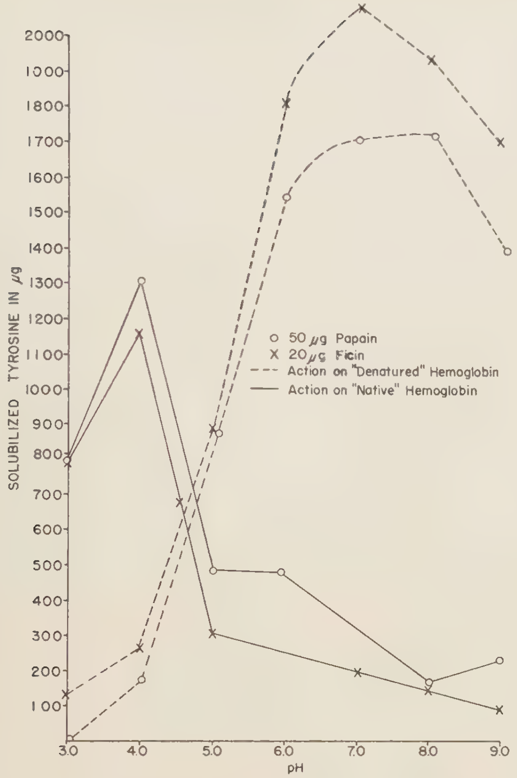

FIG. 2. pH optima of papain and ficin on urea-denatured and native hemoglobin. The ordinate plots amount of acid solubilized tyrosine released from solutions of urea-denatured and native hemoglobin buffered at the desired pH.

Collagen at pH 4.5 and below, in the presence of a low salt concentration, undergoes a visible swelling into a "gel-like" form, reversible by increasing the salt concentration or raising the pH. The physical change in collagen in the same pH range over which the plant cathepsins are active as "collagenases" suggests that an alteration in the state of the substrate is a prerequisite for the digestibility of the substrate by these enzymes. This view is supported by the observation that the proteolysis of the "gel" form of collagen by papain and ficin can be inhibited by raising the salt concentration (Fig. 3) or pH, either of which reverses the "gel" form to the native or "fibrous" form. Increasing sodium chloride concentrations over a similar range did not affect the activity of papain and ficin on hemoglobin.

On the basis of these observations, the "collagenase" action of some animal tissue cathepsins, known to be active at low pH, was studied. Beef spleen cathepsin B + C, and cathepsin C acted in a similar fashion to papain and ficin, readily digesting collagen at acid pH's with an optimum at pH 3.0. Observations on the proteolysis of native and urea-denatured hemoglobin as a function of pH by these animal cathepsins were similar to that found for the plant proteinases.

Simple watery extracts of leucocytes obtained from human purulent fluid, or from beef blood, were repeatedly observed to digest collagen at pH 1.5. Attempts to purify and concentrate this enzyme have not yet been successful.||

The possibility that the bacterial collagenase preparation also digests collagen by a two step mechanism, *i.e.* an enzymatic alteration of collagen to a digestible form followed by proteolysis, was also investigated. Direct

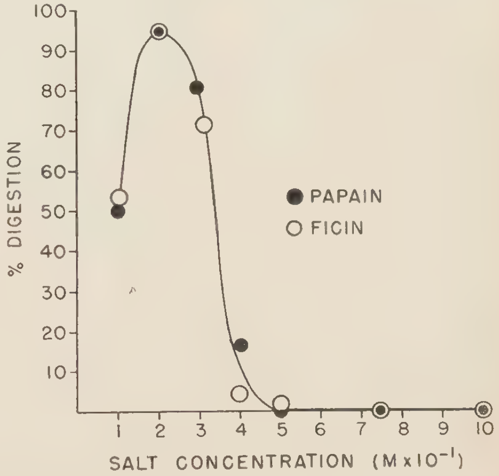

FIG. 3. Digestion of collagen at pH 3.1 by papain and ficin as a function of salt concentration. 0.1 M lactate buffer supplemented with varying concentrations of NaCl. 20 mg Achilles tendon in test. One hr incubation with 1.5 mg papain preparation; ½ hr incubation with 1 mg ficin preparation.

|| Preparations obtained by disintegrating washed leucocytes with a tuning fork or by grinding with powdered alumina were active on denatured hemoglobin over a wide pH range but did not digest collagen. The method of Tappan, Jones, and Fruton(6) on sediment of purulent exudates yielded a "leucoprotease" active only at neutrality against denatured hemoglobin but with little "cathepsin" like or "collagenase" activity.

proof of this hypothesis, namely separation of the enzymes involved, could not be achieved. A variety of fractionating procedures were not successful in producing a proteinase-free preparation capable of altering the collagen molecule, although proteolytic preparations that are unable to attack native collagen can be derived from collagenase active filtrates(8). The problem is further complicated by the presence of at least 3 different enzymes in the collagenase preparation; a cysteine inhibited collagenase, a cysteine inhibited proteinase; and a cysteine activated proteinase.

Discussion. The observations on the digestion of collagen by papain and ficin at low pH's have led us to the view that the action of the plant cathepsins on collagen is related to the state of the substrate rather than a specific characteristic of the enzymes. According to this view the dissolution of collagen in acid involves two steps: a non-enzymatic controlled equilibrium of collagen with a swollen digestible form, followed by enzymatic proteolysis. It can be shown that the extent of the non-enzymatically controlled equilibrium is determined by the pH and the salt concentration of the solution. The view of a more digestible, yet reversible, form of collagen considerably extends the number of enzymes which can degrade this substrate, for it implies that many proteolytic enzymes capable of acting below pH 4.5 may have the ability to digest collagen. The action of the plant and animal cathepsins as well as that of pepsin(4) as "collagenases" would be accounted for by this mechanism.

Observations on purulent fluids obtained from areas of active suppuration in patients, reveal the presence of a low pH with evidence of considerable proteolysis and collagen breakdown. It is suggested that in areas of suppuration, the accumulated proteinases from the autolyzed inflammatory cells, necrotic tissue, and bacterial cells contribute significantly to the accelerated collagen breakdown by the mechanism described for the cathepsins.

Summary. Papain and ficin digest collagen in the pH 2.0 - 4.5 range at low salt concentration. Evidence is presented that collagen undergoes a reversible alteration at low pH which renders it susceptible to digestion by a number of proteolytic enzymes active at acid pH's. A mechanism for the enzymatic breakdown of collagen in areas of suppuration is suggested. No evidence has been obtained to support a preliminary alteration in collagen as part of the mechanism by which *Clostridium histolyticum* collagenase preparations degrade collagen.

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Effect of Age of Rat on Choline Oxidation *in vitro*.* (21309)

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Kensler, Rudden, Shapiro and Langemann (1) reported that "choline oxidase" activity was low in the livers of young rats. These investigators did not distinguish between two of the enzymes involved in choline oxidation, choline dehydrogenase (CD) and betaine aldehyde dehydrogenase (BAD), nor was a study made of diphosphopyridine nucleotide (DPN), the coenzyme required by BAD and shown to stimulate CD activity(2,3). The work reported here was undertaken to determine which of these components was responsible for the low rate of choline oxidation by livers of young rats.

Methods. Rats of the Sprague-Dawley strain maintained on a dog chow ration were used in this study. The DPN content, choline dehydrogenase and betaine aldehyde dehydrogenase activities in the livers of these rats were determined at specified times after birth. Manometric technics were used to measure choline dehydrogenase and betaine aldehyde dehydrogenase activities. For the determination of choline dehydrogenase the method of Strength, Christensen and Daniel(3) was used with the addition of 2 μmol of DPN per flask. Betaine aldehyde dehydrogenase activity was measured by the method of Christensen(4), as follows: To the main compartment of the Warburg flask were added 1 ml of 0.1 M barbiturate buffer, pH 8.4 containing 0.1% nicotinamide; 3 μmol DPN; 18 μmol methylene blue; 14 μmol betaine aldehyde chloride; and enough water to bring the total volume to 2.6 ml. The center well contained 0.2 ml of 2 N sodium hydroxide. To the side arm of the flask was added 0.4 ml of a 30% liver homogenate in 0.1 M barbiturate buffer. After a 5-minute equilibration period at 37°C, the homogenate was tipped into the main compartment, the stopcocks closed, and the oxygen consumption measured at 10 and 20 minutes, with air as the gas phase. The time interval of 20 minutes was used to calculate oxygen uptake, since in this time the amount of betaine aldehyde formed from choline does not interfere appreciably with the determination of choline dehydrogenase activity(5). In every case, a correction was made for endogenous respiration. The choline dehydrogenase and betaine aldehyde dehydrogenase activities are expressed as microliters (μl) of oxygen uptake/hr/mg of nitrogen [Qo_2(N)]. DPN was determined by the lactic apodehydrogenase-diaphorase-methylene blue method of Sumner and Krishnan(6) with modifications as described by Strength, Mondy, and Daniel(7). DPN concentration is expressed as μg/mg of nitrogen. For each age-group, livers of rats of both sexes selected from different litters were pooled. Before pooling, each liver was divided into two portions. One portion was dropped immediately into chilled phosphate buffer and used for the DPN and choline dehydrogenase activity determinations. The other portion was dropped into the chilled barbiturate buffer and used for the determination of the betaine aldehyde dehydrogenase activity.

Three lots of animals were used for each age group. The livers from 12 animals were pooled to make one lot for the 2-day-old animals. For the one-week-old group, 9 animals were used/lot, 5 animals/lot for the 2-week-old group, 3 animals/lot for the 3-week-old group, and 2 animals/lot were used for the remaining age groups. The 6 adult animals used were from the same stock colony

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TABLE I. DPN Content, Betaine Aldehyde Dehydrogenase Activity, and Choline Dehydrogenase Activity of Rat Liver as Affected by Age of Animal.*

Age of rat	DPN content, μg/mg N	BAD† activity, Q_{O_2} (N)§	CD‡ activity, Q_{O_2} (N)
2 days	5.5	97.4	32.1
1 wk	9.5	91.9	44.9
2	15.8	90.2	50.2
3	13.8	—	58.1
5	18.9	—	65.9
7	21.9	—	71.9
9	23.8	—	75.7
13	—	—	106.6
Adult‖	25.1	107.4	125.4

* Animals from mixed litters and of both sexes were sources of tissue. Each value is avg of 3 values obtained from 3 different lots of animals, with the exception of the 5 wk results for CD which represents 2 lots.

† BAD = betaine aldehyde dehydrogenase. DPN (3 μmol) was included in the test system. No value was obtainable after the 2-wk value because of mold contamination of the betaine aldehyde chloride supply. Adult values were determined at same time as those for one-wk-old group.

‡ CD = choline dehydrogenase. DPN (2 μmol) was included in the test system.

§ Q_{O_2} (N) values are μl oxygen uptake/hr/mg nitrogen based on oxygen uptake for first 20 min. These values have been corrected for endogenous respiration.

‖ Animals were 11 mo of age from the same colony, but not the same breeding as the young rats.

but not from the same breeding. They were 11 months old when sacrificed.

Results. The data obtained on the choline dehydrogenase and betaine aldehyde dehydrogenase activities and the DPN content of livers of rats from 2 days to 13 weeks of age are summarized in Table I.

The choline dehydrogenase activity and DPN content of rat liver were low at birth, approximately 25% of the adult values, and they increased gradually with the increasing age of the animal. The DPN concentration of the liver approached the adult level when the animals were 7 to 9 weeks of age. Choline dehydrogenase activity was 85% of the adult level when the animals were 13 weeks of age. The low choline dehydrogenase activity in young rats may be related to their high requirement for choline(8). The deviation from the trend of increasing DPN concentration with increasing age of the animal, which was observed in the three-week-old rats, may have been due to an adjustment of the animal to the change in diet at the time when the young began to consume the stock ration along with the milk of the dam.

The results obtained for betaine aldehyde dehydrogenase activity indicate that the rat at birth had a betaine aldehyde dehydrogenase activity in the liver that was approximately 90% that of the adult rat. There was no significant change in the activity of this enzyme during the first 2 weeks of life. There was mold contamination in the supply of betaine aldehyde chloride making it impossible to obtain values after those for the 2-week-old group. The adult values were determined at the same time as those for the one-week-old group.

The results of this study confirm those of Kensler *et al.*(1), which indicated that "choline oxidase" activity in the livers of young rats was considerably lower than that of the adult rat. The present work suggests that this low activity may be due to a low level of choline apodehydrogenase, since betaine aldehyde apodehydrogenase was not limiting. No conclusion can be drawn with respect to the adequacy of DPN for the reactions studied, since DPN was added to the test systems. Lower concentrations of DPN were observed in the livers of young rats, however, and the possibility cannot be excluded that this factor may also be limiting in the *in vivo* oxidation of choline to betaine in young rats.

Summary. (1) The DPN content and choline dehydrogenase activity were low in livers of young rats and increased with the age of the animal. (2) The DPN content approached the adult level when the animals were 9 weeks of age. (3) Choline dehydrogenase activity reached 85% of the adult level by the time the animals were 13 weeks old. (4) The betaine aldehyde dehydrogenase activity in livers of newborn rats was 90% of that found in adult animals. (5) It appears that the low rate of oxidation of choline encountered in young rats may be due to a lack of choline apodehydrogenase. (6) The low level of DPN found in the livers of young rats may also be a limiting factor in the *in vivo* oxidation of choline to betaine.

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Loss of Pharmacologic Activity in X-Irradiated Epinephrine Solutions.* (21310)

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In the course of a study of the effects of x-irradiation of the isolated rabbit heart on its responses to epinephrine it was observed that the activity of the epinephrine solution itself, which was contained in the tubing immediately adjacent to the heart, was apparently being destroyed by the radiation. The present work was therefore carried out in order to study some of the quantitative aspects of the effect of x-rays on epinephrine solutions.

Earlier workers give conflicting reports regarding the action of x-rays on epinephrine solutions. Richter and Gerhartz(1) observed in such solutions a diminished pressor potency and a questionable decrease of blood sugar raising activity. On the other hand, Lüdin(2) saw no change in the action of x-irradiated epinephrine solutions on isolated strips of rabbit and guinea pig intestine, while Terata and Ito(3-5) reported that x-rays augmented the potency of the drug in the rabbit with respect to its inhibitory action on intestinal movements, its stimulatory action on the uterus, and its pressor effect. These latter workers noted, however, that very large doses of x-rays caused a diminution in the pressor activity of epinephrine. The discordant results reported could thus probably be readily explained by the different doses of radiation used. The actual doses employed by the earlier workers(1,2), cannot be determined in terms of modern standards. Terata and Ito(3-5) used amounts ranging from 0.25 to 5 skin erythema doses.

Materials and methods. A Keleket x-ray therapy machine was employed to deliver unfiltered radiation (250 KVP and 15 ma) to aqueous and 0.9% saline solutions of epinephrine. The solutions were placed in 25 ml portions in 1 oz polyethylene screw cap bottles and they were irradiated at a rate of approximately 470 r per minute. The bottles were arranged horizontally in a compact circle 28 cm from the target and were slowly rotated on a revolving platform while being exposed. Dogs were used for the bioassay of the pressor activity of some of the solutions by the method described in the U.S. Pharmacopeia XIV. Qualitative tests for epinephrine activity were made on the isolated perfused rabbit heart (Langendorf method) and on the isolated rabbit ileum suspended in Locke-Ringer solution. Estimations of the epinephrine content in irradiated solutions were also made by a modification of the fluorometric procedure of Lund (5). In this method epinephrine is oxidized to adrenochrome by manganese dioxide and further changed to fluorescent adrenolutine by sodium hydroxide. The amount of fluorescence can be determined photo-electrically and

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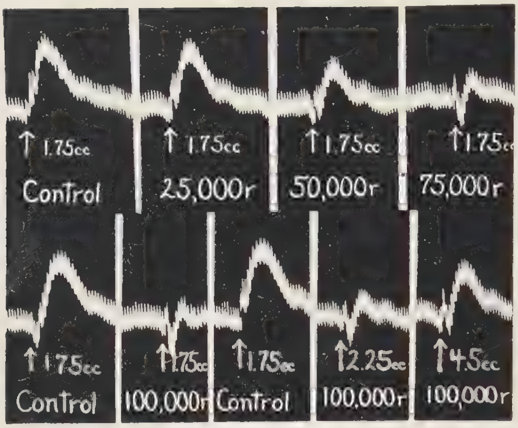

FIG. 1. Blood pressure responses of a 13 kg female dog to a 1:100,000 dilution of epinephrine-HCl previously irradiated with various doses of X-ray. Control responses are approximately 40 mm Hg increases in blood pressure.

is in direct proportion to the amount of adrenolutine present. For the present purpose it was possible to simplify the method because the starting material was a simple aqueous or saline solution. Satisfactory results were obtained by adding 0.1 g of manganese dioxide directly to 10 ml portions of the properly diluted sample in centrifuge tubes. A Corning 5874 filter plus a perforated metal screen to exclude partially the ultraviolet light was used as the primary filter and a Corning 3486 was employed as the secondary filter. This filter combination gave a full scale reading on a Coleman Model 12B Electronic Photofluorometer corresponding to 1 μg/ml.

Results. A semi-quantitative demonstration of the decrease in pharmacologic action of x-irradiated epinephrine solutions is provided by the results illustrated in Fig. 1. The blood pressure response of a dog decreased progressively as the dose of x-ray delivered to the drug solution increased from 25,000 to 100,000 r. More than 60% loss of pressor activity was seen in the solution receiving 100,000 r. Further qualitative demonstration of this effect was seen in the isolated rabbit heart and ileum which failed to respond to doses of irradiated (100,000 r) epinephrine (1:100,000) which, in the non-irradiated form, elicited strong responses in these preparations.

A quantitative comparison of the results of dog pressor and fluorophotometric assays was made to ascertain the degree of parallelism between the pharmacologic and chemical deterioration in x-irradiated epinephrine solutions. The extent of destruction in solutions receiving 50,000 r is shown in Table I. A close agreement exists between the results of the two methods of assay. This furnishes some evidence against the formation of products having cardiovascular activity. The solution containing sodium bisulfite showed somewhat less destruction of epinephrine indicating a slight protective action of this preservative in the concentration used.

Fig. 2 shows the exponential type of decay observed when both aqueous and saline solutions of epinephrine-HCl§ were subjected to massive doses of x-ray. These analyses were performed by fluorophotometric assay. It may be seen that the more dilute the solution the greater was the per cent destruction, and that at each dilution the epinephrine in saline solution was consistently less affected than that in aqueous solution. In this type of assay it was necessary to consider the possibility that a quenching effect of the sodium chloride might influence the differences observed between the saline and aqueous solutions. An example of this effect is given by Lund(5) who found proportionate decreases in the intensity of fluorescence of adrenolutine in the presence of increasing amounts of ascorbic acid over 0.4 mg/ml. Tests performed on equal concentrations of epinephrine in water and 0.9% NaCl, however, gave almost identical readings for the two solutions, thus indicating that the

TABLE I. Assays of Epinephrine Solutions after 50,000 r X-Irradiation.

	% destruction	
Drug (1:100,000 aqueous dilutions)	Dog pressor assay*	Fluorophotometric assay
l-Epinephrine-HCl + 20 μg/ml $NaHSO_3$	64 ± 2	64
Epinephrine-HCl†	—	82
l-Epinephrine bitartrate	76 ± 2	78

* Official USP XIV assay (including stand. error of assay) for epinephrine.

† Prepared from refined crystalline epinephrine obtained from natural sources and containing small amounts of nor-epinephrine.

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§ Refined crystalline epinephrine provided through the courtesy of Dr. M. C. Ziporyn of the Armour Laboratories.

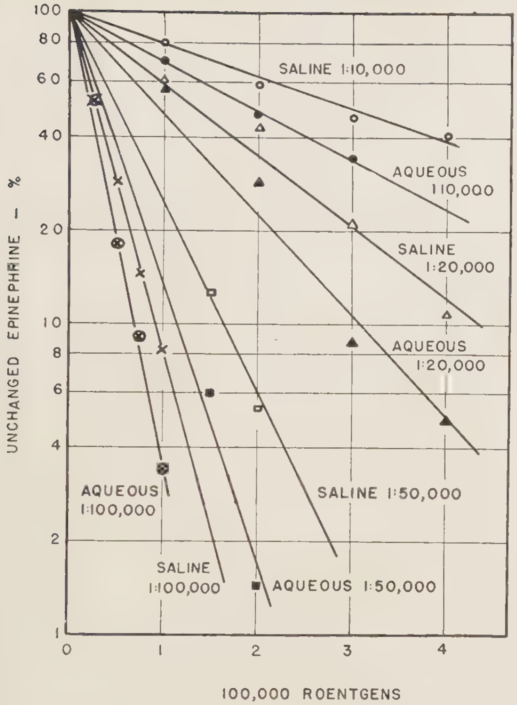

FIG. 2. Destruction of aqueous and 0.9% saline solutions of epinephrine-HCl by X-rays.

presence of 0.9% NaCl had no influence on the fluorophotometric estimation of epinephrine.

When solutions of epinephrine are allowed to remain exposed to light and in contact with air, a pink coloration of the solution soon develops due to the presence of adrenochrome, an oxidation product of epinephrine. No pink coloration developed, however, in the irradiated solutions which showed the diminished pharmacologic activity discussed above. Furthermore, adrenochrome could not be detected spectrophotometrically in these solutions.

The results presented here show that solutions of epinephrine lose their pharmacologic activity when x-irradiated. Qualitative and quantitative biological evidence of such loss of activity is seen in the absence of typical responses of the isolated rabbit heart and intestine to the irradiated solutions and in the diminution of the blood pressure rise in the anesthetized dog. Contrary to the findings of Terata and Ito(4), we found no evidence of an augmentation of the pharmacological activity of epinephrine solutions as a result of exposure to x-ray.

That a chemical change takes place when aqueous or 0.9% saline solutions of epinephrine are exposed to x-rays is apparent when the irradiated solutions are subjected to fluorometric assay. By means of the latter analytical method it was possible to show that 0.9% NaCl exerts a moderate preservative action against the action of x-rays on solutions of epinephrine.

It seems probable that the products resulting from the action of x-rays on epinephrine possess little or no cardiovascular activity as demonstrated by the close agreement of the results of the dog pressor assay and the fluorometric assay. Adrenochrome could not be detected as one of the products of the chemical change.

Considering the relatively large doses of x-ray needed to reduce the pharmacologic activity of the epinephrine solutions or to destroy epinephrine chemically it would appear unlikely that any of the effects of irradiation of the intact animal by doses of practical significance could be accounted for by the destruction of endogenous epinephrine.

The results of this study provide one example of the deterioration which a drug may undergo when exposed to x-ray. The possibility should be considered that other drugs may be changed or undergo decomposition during irradiation sterilization or in experimental work involving the incidental exposure of drugs to x-ray.

Summary. Epinephrine in dilute solution is destroyed by x-irradiation to yield what appear to be pharmacologically inert decomposition products. The presence of 0.9% sodium chloride offered a moderate protective action against this effect. The extent of loss of pharmacologic activity of epinephrine is exponentially related to the dose of x-irradiation.

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Protective Effects of Alfalfa in Immature Mice Fed Toxic Doses of Glucoascorbic Acid.* (21311)

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Woolley and Krampitz(1) have observed that immature mice fed a purified ration containing 5 to 10% glucoascorbic acid developed a severe condition characterized by failure of growth, diarrhea, subcutaneous hemorrhages, an unthrifty appearance, alopecia and death. These effects did not occur in mice fed similar doses of glucoascorbic acid in conjunction with a natural food ration. Supplements of ascorbic acid when added to the purified ration were without protective effect. The deleterious effects of glucoascorbic acid in mice fed the purified diet were counteracted, however, by the concurrent feeding of dried grass (Cerophyl). In the present communication data are presented indicating that desiccated alfalfa is also effective in counteracting glucoascorbic acid toxicity in mice fed a purified ration and that the protective factor therein is apparently distinct from any of the known nutrients.

Procedure. The basal ration employed in the present experiment consisted of cerelose, 63%; casein,† 24%; salt mixture,‡ 5%; cellulose,§ 2%; cottonseed oil, 2%; and glucoascorbic acid, 4%. To each kg of the above diet were added the following synthetic vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg, 2-methyl-naphthoquinone, 5 mg; and choline chloride, 2 g. To each kg of the above diet were also added 5000 U.S.P. units of vit. A, 500 U.S.P. units of vit. D and 30 mg of alpha-tocopherol acetate. The vitamins were added in place of an equal amount of cerelose. In addition to the basal ration the following diets were also employed, consisting of the basal ration plus each of the following supplements: (a) 10% oven dried alfalfa, (b) 20% oven dried alfalfa, (2) 2.5 alfalfa ash, (d) 10% casein, (e) 12% cellulose, (f) supplements of the following vitamins/kg of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg; and 2-methyl-naphthoquinone, 5 mg, and (g) supplements of the following fat-soluble vitamins/kg of ration: vit. A, 5000 U.S.P. units, vit. D, 500 U.S.P. units; and alpha tocopherol acetate, 270 mg. Supplements were added in place of an equal amount of cerelose. In addition to the above rations a control diet was also tested which consisted of the basal ration with glucoascorbic acid omitted and replaced by an equal amount of cerelose. Ninety male mice of the Webster strain ranging from 11 to 14 g in weight were employed in the present experiment. Animals were placed in metal cages with raised screen bottoms and were fed the above diets *ad lib* (10 mice per group). Animals were fed daily. All food not consumed 24 hours after feeding was discarded. All diets were stored under refrigeration. These measures were employed to minimize oxidative changes in the diet. Feeding was continued for 12 days or until death, whichever occurred sooner.

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* Communication No. 358 from Department of Biochemistry and Nutrition, University of Southern California.

This investigation was supported in part by a grant-in-aid from Nutrilite Products, Buena Park, Calif. The author is indebteded to Mr. Carl F. Rehnborg of Nutrilite Products, Inc., for providing the oven dried alfalfa and the alfalfa ash and to Dr. Philip P. Gray of the Wallerstein Laboratories, New York City, for supplying the glucoascorbic acid employed.

† Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, O.

‡ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, O.

§ Solka Floc, Brown and Co., Boston, Mass.

TABLE I. Effects of Desiccated Alfalfa and Supplements of Known Nutrients on Weight Increment of Immature Mice Fed Toxic Doses of Glucoascorbic Acid (8 Animals per Group).

Supplements fed with basal ration	Avg change in body wt during exp.* (g)	Symptoms
None	− 1.8 ± 0.4 (8)	Severe
10% oven dried alfalfa	+7.2 ± 0.8 (8)	0
20% *Idem*	+7.3 ± 0.5 (8)	0
2.5% alfalfa ash	− 0.7 ± 0.3 (6)	Severe
10% casein	− 2.4 ± 0.4 (6)	”
12% cellulose	+4.2 ± 0.4 (8)	0
B vit., ascorbic acid and 2-methyl naphthoquinone	− 1.2 ± 0.5 (4)	Severe
Vit. A, D and E	+0.1 ± 0.3 (8)	”
Basal ration without glucoascorbic acid	+8.7 ± 0.7	0

* Including stand. error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the No. of observations.

Values in parentheses indicate No. of animals which survived and on which averages are based.

Results. Findings are summarized in Table I. Data were computed on the basis of the top 8 animals in each group to minimize variations in averages due to early deaths and atypical responses on the part of individual mice. Findings on the basal ration confirm those previously reported by Woolley and Krampitz(1) for mice fed a purified diet containing 5% glucoascorbic acid. These include loss in weight, diarrhea, an unthrifty appearance, irritation and swelling of the anal region, and subsequent to the first week of feeding, alopecia. By the seventh day of the experiment symptoms were well advanced. These effects were completely counteracted by the addition of dried alfalfa to the basal ration at a 10 or 20% level in the diet. Alfalfa ash at a level corresponding to the amount provided by 20% dried alfalfa in the diet was without protective effect. Increasing the casein content, doubling the B vitamin, ascorbic acid and 2-methyl naphthoquinone content, or doubling the vit. A and D and increasing 10-fold the alpha-tocopherol acetate content of the basal ration were also without protective effect. Purified cellulose when incorporated in the basal ration at a 12% level completely counteracted all symptoms of glucoascorbic acid toxicity. The weight increment of mice on this ration, however, was significantly less than that of mice fed the dried alfalfa or the basal ration with glucoascorbic acid omitted.

Discussion. Findings indicate that the deleterious effects observed in mice fed a purified ration containing 4% glucoascorbic acid were completely counteracted by the concurrent feeding of dried alfalfa. The protective effects of alfalfa were not due to its mineral content or to its content of any of the known vitamins. Additional protein *per se* provided by the alfalfa does not appear to be the protective factor. Subsequent findings indicate that increasing the fat content of the basal ration to 10% in the form of cottonseed oil was also without protective effect. Significant protection was obtained, however, by the addition of purified cellulose to the basal ration. It is possible, therefore, that the protective effects of dried alfalfa were due, at least in part, to its crude fiber content. On the other hand, the beneficial effects of cellulose may have been due not to this material *per se* but to its favorable influence on the synthesis by the intestinal flora of a protective factor (or factors) similar to that present in the dried alfalfa. Since the weight increment of the mice fed dried alfalfa was significantly greater than that of the mice fed purified cellulose, it appears that alfalfa contains some factor (or factors) other than cellulose or the nutrients indicated above which under conditions of the present experiment had growth-promoting activity in mice fed toxic doses of glucoascorbic acid.

Summary. Immature mice fed a purified ration containing 4% glucoascorbic acid exhibited loss in weight, diarrhea, an unthrifty appearance, irritation and swelling of the anal region and alopecia. These effects were completely counteracted by the addition of dried alfalfa to the purified ration at a 10% level

in the diet. The protective effects of alfalfa were not due to its mineral content or to its content of any of the known vitamins. Supplements of casein or cottonseed oil were without beneficial effect. Purified cellulose completely counteracted all symptoms of glucoascorbic acid toxicity; the weight increment of mice fed this supplement, however, was significantly less than that of animals fed dried alfalfa.

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Influence of Nortestosterone Cyclopentylpropionate on Erythropoiesis in Castrate Male Rat. (21312)

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From the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

The discoveries that 19-nortestosterone has relatively more myotrophic activity than sex-accessory-stimulating activity, when compared with testosterone(1), and that the cyclopentylpropionate ester of 19-nortestosterone (NTCP) is more potent than the unesterified compound(2) have led us to a broad investigation of the biological properties of NTCP to find whether its difference from testosterone is limited to the androgenic phase of its activity or whether there are other divergencies in the biological spectra of the two compounds. One of the interesting properties of testosterone is its ability to stimulate the formation of red blood cells(3). This effect has been noted in castrated(3) and hypophysectomized(4,5) rats as well as in man, where testosterone stimulates hemopoiesis in eunuchoidal(6), hypopituitary, and hypothyroid patients(7). The mechanism for the erythropoiesis-stimulating properties of testosterone has not been defined, although it is possible that it is linked to its well-known protein anabolic activity. Growth hormone, an entirely different anabolic agent, also causes stimulation of the hemopoietic tissues(8).

Therefore, in an effort to continue our comparison of NTCP with testosterone and to cast more light on the role of anabolic-androgenic compounds in red blood cell formation, we investigated the ability of NTCP to cause erythropoiesis in the castrate rat. It was concluded that NTCP, like testosterone, does possess this property. This suggests that the blood-forming activity is a function of the general metabolic action of the androgens rather than of the sex-accessory-stimulating action.

Methods and materials. Eighteen male Sprague-Dawley rats were used in the experiment. They were castrated at a mean weight of 150 g, then maintained without treatment for 11 mos. At the beginning of the observation period all the animals were apparently healthy and weighed a mean 417 g (378-460). During the 7-week experimental period they were fed stock laboratory ration *ad libitum* and were caged 2 or 3 per 8″ x 11″ x 7″ cage. Erythrocyte counts and hemoglobin (Hb) determinations were made on each rat once during weeks 1, 2, 5, 6, and 7. The blood studies were done on Wed., Thurs., and Fri. of these weeks and in most cases values for any rat were obtained on the same day in successive weeks. Weeks 1 and 2 served as a control period; *i.e.*, before institution of steroid treatment. Since testosterone propionate (TP) has been used in previous hemopoiesis experiments, both experimentally and clinically, we chose this ester for a standard in the evaluation of NTCP. The rats were divided into 5 groups of 3 or 4 per group and injected subcutaneously once daily with .1 ml cottonseed oil containing (A) no steroid, (B) TP .25 mg, (C) TP 1 mg, (D) NTCP .25 mg, or (E) NTCP 1 mg. Injections were begun in the middle of week 3 and continued until the end of week 7. Cell counts and Hb determinations were made on tail blood obtained by warming the tail in 45° water for about 30 seconds, then

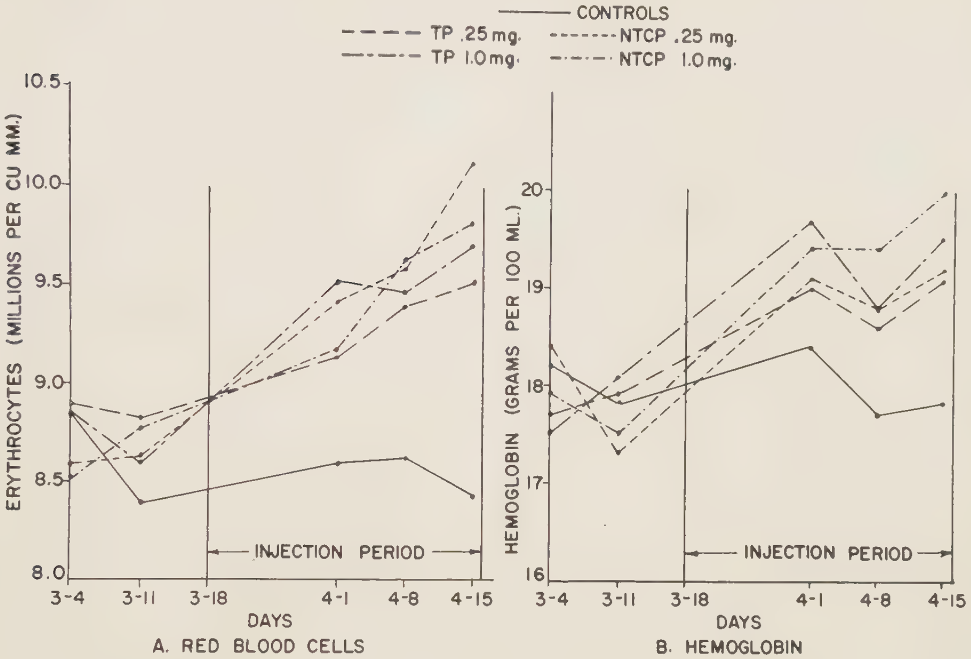

FIG. 1. Changes in red blood cell count (A) and in hemoglobin concentration (B) in castrate rats treated with TP and NTCP.

slicing off a small portion of the tip with a razor blade. Hemorrhage after blood withdrawal was prevented when necessary by application of a dressing of *Gelfoam*.* Blood was drawn into a certified RBC diluting pipette to the third graduation, diluted with Hayem's solution, shaken, and counted in a certified Spencer Bright-Line counting chamber. Two samples were counted for each rat at each bleeding. Variance between duplicate samples never exceeded ±2.2%. Hb was estimated by the acid hematin method(9). Body weights were recorded at weekly intervals throughout the experiment. On the 4th day following the last injection the rats were chloroformed and the seminal vesicles were removed and weighed.

Results. The data are shown in Fig. 1. Progressive rises in RBC count and Hb concentration occurred in all steroid-treated rats. All groups gained weight during the treatment period. The low doses of TP and NTCP caused weight gains of 46 and 33 g respectively, compared with 20 g in controls, but the high doses caused inhibition of weight gain (19 and 3 g increases respectively). This inhibition of weight gain at elevated doses has been noted before(10) but is unexplained.

The steroids induced marked hemopoietic responses, but the differences between doses and compounds are not considered significant. When the experiment was devised it was believed that the interval between low and high dose of the steroids was sufficiently great for differential responses to be obtained. At the end of the experiment it was obvious that both doses produced responses that may be considered the same within the limits of experimental error.

All doses used were sufficiently high and were given for a long enough period to exert marked androgenic effects, as indicated by seminal vesicle weights. The oil-injected controls had seminal vesicles of 16 mg; the high doses of TP and NTCP produced seminal vesicles of 531 and 592 mg respectively and the low doses 400 and 294 respectively. The previously established differential in androgenic potency of the two compounds(2)[†] is still apparent at the lower doses, but at the 1 mg dose, maximal stimulation was obtained with both compounds.

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* Registered Trade-mark, Upjohn Co., Kalamazoo, Mich.

The progressive rise in RBC count throughout a 27-day injection period is of particular interest when one considers that in the force-fed rat(10) as well as in the *ad libitum*-fed rat(11) the protein anabolic effect (nitrogen retention) of testosterone disappears in about half this time. If the hemopoietic effect of anabolic-androgenic compounds is to be linked to the nitrogen-retaining effect, it must be postulated that the impetus to the blood-forming tissues given by the anabolic compound in the first two weeks is sufficient to cause continued formation and release of blood cells for some time after the nitrogen-retaining effect of the steroid has disappeared.

The important conclusion that is drawn from these data is that NTCP, a steroid biologically resembling TP in some respects, yet differing from it in others(2,10) is similar to TP with respect to its ability to stimulate erythropoiesis in the rat.

Summary. 1. Long-time castrated male rats had a mean circulating red blood cell count of 8.70 ± .43 million cells/cu mm before treatment. Treatment with testosterone propionate (TP) or nortestosterone cyclopentylpropionate (NTCP) at daily doses of .25 mg or 1 mg for 27 days increased the number of cells to group averages of 9.52 to 10.14 million/cu mm. Blank-injected controls had RBC counts averaging 8.42 million at the end of the experimental period. 2. Hemoglobin concentration rose from a pretreatment mean of 17.88 g/100 ml to group averages of 19.1 to 20.0 g/100 ml during steroid treatment. Blank-injected controls had 17.8 g/100 ml at the end of the treatment period. 3. It is concluded that nortestosterone cyclopentylpropionate is similar to testosterone propionate in causing increases in red cell count and Hb in the castrate rat.

[†] Five multi-dose assays have been done, comparing seminal-vesicle-stimulating potency of NTCP and TP at doses from 12.5 to 500 μg/day in weanling rats. NTCP has about 20% the androgenic activity of TP under these conditions.

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Induction of Mammary Growth in Rats by Estrogen and Progesterone.*† (21313)

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In a previous communication(1) we reported analysis of the nucleic acids of mammary glands from normal rats during pregnancy lactation and involution. It was shown that both the desoxyribonucleic acid (DNA) and pentosenucleic acid (PNA) content of the glands increased during pregnancy when lobule-alveolar growth occurs and that pentosenucleic acid increased further with increasing lactation.

In the present study attempts were made to induce mammary growth by the administration of hormones to rats under various conditions and to determine growth of the mammary glands both by nucleic acid analyses and by histological examination.

Materials and methods. Female albino rats weighing about 170 g at the start of the experimental period were used in all cases. Castrated rats were allowed a 10 day recovery period before analysis or administration of hormones. The 6 posterior glands were removed and the nucleic acids determined as previously described(1). Pectoral glands were fixed and stained for histological examination.

Experimental. Castration followed by a 10-day recovery period resulted in a decrease in the nucleic acid content of the mammary glands as compared to the virgin (Table I). Histologically these glands were reduced to a smaller area and showed a thin duct system without end-buds demonstrating the regressed state of the gland. Castrate rats treated for 21 days with progesterone and estradiol-benzoate in ratios of 1, 5, and 10 mg of progesterone to 1 μg of estradiol-benzoate (in olive oil) yielded glands whose DNA and PNA values increased with the increasing ratio of the 2 hormones (Table I). The lowest ratio gave no increase over the virgin condition. The 2 higher ratios gave lower values, with high individual variation, than normally found in the 12-15 day pregnant rat. Glands resulting from the administration of the 2 higher ratios showed a well developed lobule-alveolar system somewhat limited in its peripheral extension.

Progesterone and estradiol-benzoate (5 mg : 1 μg) were administered to castrate rats for periods of 15, 21, 25 and 32 days to determine the time and extent of gland growth. Results of nucleic acid analyses show glands of each group had growth (DNA) equivalent to glands of normally pregnant and lactating rats. Glands from rats receiving hormone treatment for 15 days showed good duct and lobule development while longer treatment periods produced definite secretory engorgement of the lobule-alveolar system. PNA values for these glands were found to be comparable to PNA values of glands near midpregnancy. As such they were much lower than those previously reported for lactating rats(1).

Castrate - hypophysectomized rats were treated for 21 days with 5 mg progesterone and 1 μg estradiol benzoate. The mammary epithelium of these glands consisted of an extremely fine tubule system with no end buddings. The DNA and PNA content of these glands was very low.

Results. The role of the ovarian hormones in mammary development has received extensive study(2). Our results show that removal of the ovaries causes a decrease in mammary epithelium as seen by the decreased DNA values. This brings the mammary gland complex to a state in which almost the total amount of DNA is contributed by fat and connective tissue. It is from this regressed state that epithelial growth should be readily demonstrable by the use of DNA analyses. The

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* Contribution from the Department of Dairy Husbandry, Mo. Agric. Exp. Station, Journal Series No. 1454 Approved by the Director.

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‡ Postdoctoral Fellow of the National Cancer Institute.

TABLE I. Nucleic Acid Values for Rat Mammary Glands.

Treatment	Days	No. of animals	DNA, mg/6 glands	PNA, mg/6 glands
Virgin		5	5.7 ± .4	3.5 ± .4
Castrates		6	3.1 ± .4	2.4 ± .5
Normal pregnant	10–18	7	12.4 ± .7	11.8 ± 2.4
Castrates, P-E,* 1 mg : 1 μg	21	5	4.2 ± .7	4.1 ± 1.4
Castrates, P-E, 5 mg : 1 μg	21	6	7.6 ± 3.0	9.2 ± 5.0
Castrates, P-E, 10 mg : 1 μg	21	6	9.2 ± 2.8	10.6 ± 4.0
Castrates, P-E	15	5	12.9 ± 1.5	10.3 ± 1.4
	21	5	14.5 ± 1.5	12.4 ± 2.3
	25	4	13.2 ± 2.1	10.2 ± 2.4
	32	5	15.4 ± 2.1	10.2 ± 3.8

* P-E = Progesterone-estradiol benzoate at 5 mg to 1 μg unless otherwise specified.

use of nucleic acid as an indication of mammary gland development is admittedly crude and subject to considerable error but we believe it can be used in conjunction with histological examination for a quantitative description of conditions in the glands.

In the rat, various investigators have attempted to find the optimum progesterone-estrogen synergism for mammary growth. Astwood and Geschicter(3) reported a dosage of 1 mg of progesterone and 5 μg of estrone daily for 8 days induced no demonstrable lobule-alveolar growth. Selye(4) reported good lobule development in castrate rats with 15 mg of progesterone administered for 10 days. Meites and Turner(5) reported limited lobule-alveolar development in the castrate rat receiving 15 mg of progesterone and 50 μg estrone daily for 10 days. Lyons and co-workers(6,7) have used 4 mg of progesterone and 1 μg estrone in their studies of mammary gland development. Curtiss(8) found no effect of 15 mg of progesterone alone for 12 days but .1 mg estradiol twice weekly plus 4 mg progesterone daily gave optimal growth. The work of Elliott and Turner(9) using the "mammary spreading factor" as an indicator of mammary growth demonstrated a ratio of 5 mg progesterone to 1 μg estradiol-benzoate as optimal.

The data in Table I show that 5 mg progesterone and 1 mg estradiol can promote lobule-alveolar growth in castrate rats equivalent to that of normal and lactating rats. Such levels of growth were not obtained with this ratio in all experiments as seen by the lower DNA values of one group treated for 21 days. Though these particular results are unexplained numerous subsequent experiments have demonstrated good mammary growth with this ratio. Prolonged administration of the progesterone-estrogen treatment did not further stimulate lobule-alveolar growth but the glands showed a lactational condition when examined histologically. In other experiments it was found that when castrate rats were treated for 26 days and all treatment stopped, they came into lactation sufficient to nourish foster litters.

Administered progesterone and estrogen apparently provide the stimulus for proliferative growth of the mammary epithelium. The growth rate declines as the gland reaches normal size and slight secretory activity of the gland sets in with no modification in the progesterone-estrogen treatment. PNA values indicate that the secretory level of the glands is low, being comparable to the mid-pregnant rat.

The involvement of the pituitary in mammary development is again confirmed by the inability of the synergistic ratio of progesterone and estrogen to elicit mammary development in castrate-hypophysectomized rats.

Summary. The administration of estrogen and progesterone to castrate female rats for 15 days or more stimulated an increase of DNA comparable to that found at a corresponding period of pregnancy. Continued injection of these hormones for periods up to 32 days increased only slightly the DNA content. This is interpreted as indicating a cessation of further gland proliferation even in the presence of these hormones. In the hormone stimulated glands the PNA content increased comparable to pregnancy but since profuse lactation was not stimulated it did not increase compared to the latter stages of pregnancy or lactation.

However, when the hormones were administered for 26 days and then stopped, lactation set in sufficient to nourish foster litters of rats.

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Isolation and Identification of Aldosterone from Adrenal Venous Blood.* (21314)

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A highly potent sodium-retaining factor has been isolated from adrenal extracts by Grundy, Simpson and Tait(1). These investigators in collaboration with Reichstein and Wettstein and their collaborators(2) have recently isolated this material in pure form and established its structure. The same compound has recently been obtained in pure crystalline form by Mattox, Mason and Albert(3). The presence of an aldehyde group at C-18 has led to the name "aldosterone" for the new compound.

The isolation of aldosterone from adrenal tissue naturally stimulated investigators to search for this compound in the effluent blood of this gland. Fractions characterized by their ability to induce sodium retention in very small doses have been isolated from adrenal venous blood by two groups of workers(4-6). The present study is concerned with the further characterization of this material. The observations here recorded make it highly probable that the product isolated from adrenal venous blood is essentially homogenous and identical with aldosterone.

Methods. Male mongrel dogs were anesthetized with sodium pentobarbital and hypophysectomized by the oral approach. Six hours after hypophysectomy, the left lumboadrenal vein was cannulated for collection of adrenal venous blood, the adrenal vein ligated, and sufficient heparin infused to prevent clotting. The blood was collected in vessels kept at 0°. A volume of 1946 ml of adrenal venous blood was collected from 6 hypophysectomized animals. An additional 2345 ml of adrenal venous blood was collected from 4 hypophysectomized animals subjected to the same experimental conditions except that 100 U of ACTH[‡] were infused during the collection period. The adrenal venous blood was diluted 1:1 with water and extracted twice with a volume of chloroform equal to the blood-water mixture. The residues of the chloroform extracts were partitioned between 70% ethanol and hexane. The solutes obtained from the 70% ethanol fractions were chromatographed 72 hours on paper in the system propylene glycol-toluene(7). The region of

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[†] National Science Foundation Predoctoral Fellow.

[‡] Wilson "Corticotropin Solution", 100 U in 200 ml 0.9% NaCl solution made to 0.01 N with HCl.

each chromatogram corresponding to the position of cortisone, previously shown to contain the active sodium-retaining factor(1,5) was eluted with methanol, taken to dryness *in vacuo* and acetylated (acetic anhydride:pyridine 1:2 for 16 hours at 25°). The acetylated products were chromatographed on paper 72 hours in the system propylene glycol-hexane. Several fractions were found on analysis of the chromatograms by ultraviolet spectroscopy. Aliquots of each fraction were subjected to hydrolysis with acetylcholinesterase in glycylglycine buffer (pH 7.4 for 4 hours at 30°) and assayed for sodium-retaining activity in adrenalectomized rats.§ The fraction of each chromatogram which migrated at a rate slightly less than that of 11-dehydrocorticosterone acetate yielded highly active material on hydrolysis. The active fractions, one from hypophysectomized dogs, the other from hypophysectomized ACTH-treated dogs, were rechromatographed separately for 96 hours in the propylene glycol-hexane system. An apparently homogeneous substance was found 18 cm from the origin of each chromatogram. Quantities equal to 78.7 and 73.8 μg were obtained from adrenal venous blood of the hypophysectomized and the hypophysectomized ACTH-treated dogs, respectively. Estimates are based on $\epsilon = 15850$ for absorption at 240 mμ(8,9). An aliquot of each substance was hydrolyzed with acetylcholinesterase and assayed for sodium-retaining activity. Each sample was found to be highly active; a statistically valid difference in potency did not exist between the sample obtained from the hypophysectomized animals and that from the hypophysectomized animals treated with ACTH. The two samples when combined and chromatographed in the system half-strength propylene glycol-hexane for 22 hours appeared as a single component 6.5 cm from the origin. A sample of aldosterone obtained from Doctors Mattox and Mason was acetylated and chromatographed in the same tank. The diacetate of this preparation had migrated to the same position as that of the acetate from adrenal venous blood.

The sample from adrenal venous blood was dissolved in carbon disulfide and examined in the infra-red employing a micro-cell of new design. The infra-red absorption spectrum was identical with that of the diacetate of authentic aldosterone. The sample from adrenal venous blood and a sample of authentic aldosterone diacetate were hydrolyzed with acetylcholinesterase. The hydrolysis products were chromatographed in the system half-strength propylene glycol-toluene for 15¼ hours; on each chromatogram a single component was found which migrated at a rate characteristic of free aldosterone. As estimated by analysis in the ultraviolet, 18% of the substance from adrenal venous blood and 25% of the reference substance from adrenal tissue was recovered in this fraction. Each substance was bioassayed for sodium-retaining activity at doses of 0.027, 0.108 and 0.432 μg. The substances were highly active and equipotent. The ratio of potency of the substance from adrenal venous blood to that of the authentic aldosterone was $1.2 \pm .45$ ($\lambda = 0.52$).

Overflows from the chromatograms of the hydrolysis products contained approximately 75% of the starting material. The overflows were rechromatographed in the system half-strength propylene glycol-hexane for 78 hours; on each chromatogram a single component had migrated to 5 cm from the origin. 11-Dehydrocorticosterone had migrated to 10 cm from the origin. In view of the relatively high polarity of the overflow substance as compared to the known diacetate, it was considered to be a monoacetate. On hydrolysis and rechromatography in propylene glycol-toluene a substance equivalent in quantity to about 20% of that in the hydrolysis mixture migrated at a rate characteristic of free aldosterone. The substance was again bioassayed for sodium-retaining activity at doses of 0.027, 0.081 and 0.243 μg, and found to have a potency of 48 ± 16 ($\lambda = 0.40$) times that of desoxycorticosterone acetate.

An aliquot of the monoacetate in concentrated sulfuric acid (25°, 3 hours) was examined spectroscopically over the range 220 to 500 mμ. A single large maximum at 285 mμ was found, with a small shoulder at 390 mμ. A sample of monoacetate of authentic aldosterone, examined simultaneeously under the same conditions, exhibited an identical absorption spectrum.

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§ Na and K excreted in a 5-hour period following steroid administration are analyzed by flame photometry. The response is expressed as the ratio Na/K.

Discussion. The comparison of the product isolated from adrenal venous blood with aldosterone has demonstrated 1) identity of the infra-red spectrum of the acetylated products, 2) identical chromatographic mobility of the parent compound and of two derivatives, 3) equal potency in inducing sodium retention and 4) identical spectrographic properties (the ultraviolet maximum at 238.5 mμ, the maximum at 285 mμ with a shoulder at 390 mμ shown in concentrated sulfuric acid, as well as the negative Porter-Silber test, have been reported previously(5,6) and have been confirmed in the present study.) It is very probable, therefore, that the two products are identical and that the aldosterone elaborated by the adrenal gland is secreted unaltered into the blood stream.

The biological activity of the aldosterone isolated from adrenal venous blood was found by our assay method to be 48 ± 16 times that of desoxycorticosterone acetate, a potency somewhat less than that reported by other workers(3,8). This difference is not considered to mean that the substance isolated from adrenal venous blood is less potent than aldosterone from adrenal tissue. Inherent variability of the assay method and minor differences in technic probably explain the apparent discrepancy. Furthermore, direct comparison of aldosterone and the substance from adrenal venous blood by the assay method employed in this laboratory indicates these substances to be equipotent.

One of the samples of aldosterone isolated in this study was obtained from dogs 6 to 10 hours after hypophysectomy at which time the rate of secretion of other adrenal steroids was markedly reduced. Analysis of 17-hydroxycorticosterone, corticosterone and other Δ^4-3-ketosteroids in this blood showed their rate of secretion to be about 1/10 that of sham-operated dogs. Aldosterone was the only steroid present in an amount capable of exercising significant metabolic effects. The administration of ACTH markedly increased the concentrations of 17-hydroxycorticosterone, corticosterone and other Δ^4-3-ketosteroids but influenced the secretion of aldosterone only slightly, if at all. These observations are of great interest but should be considered preliminary. Definitive statements as to the effects of hypophysectomy and ACTH administration on aldosterone secretion must await more extended and more detailed experimental study of this problem.

Summary. A highly potent sodium-retaining steroid has been isolated from adrenal venous blood and demonstrated to be identical with aldosterone with regard to: 1) infra-red spectrum, 2) chromatographic behavior of the parent compound and 2 derivatives, 3) biological activity in inducing sodium retention in adrenalectomized rats, 4) ultraviolet absorption and 5) sulfuric acid chromogen.

The authors wish gratefully to acknowledge gifts of aldosterone from Doctors Vernon Mattox and Harold Mason and from Dr. A. Wettstein, and the advice and aid of Dr. Ernest Bueding in the development of the enzymatic hydrolysis procedure. We wish also to acknowledge the valuable technical assistance of Mr. Robert C. Banks.

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ACTH-Suppressing Action of Aldosterone.* (21315)

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The adrenal hormone, aldosterone, formerly called electrocortin, has recently been crystallized(1,2) and its formula established(3). It is much more potent than desoxycorticosterone in affecting electrolyte metabolism(4,5) and as adrenal substitution therapy in the dog(6-8) and man(9,10). It also possesses anti-stress (11) and other cortisone-like properties(12, 13).

The possibility has been mentioned frequently that aldosterone might be the life-maintaining hormone produced by the adrenal after hypophysectomy and, hence, more or less independent of the pituitary. If that were true, it might, unlike cortisone, be ineffective as an inhibitor of ACTH release. In any case, the question of whether an adrenal hormone does or does not affect ACTH release has important theoretical and therapeutic implications. It was with such considerations in mind that the experiments reported here were undertaken.

Methods. The indirect method used to measure the presumed ACTH-suppressing activity of aldosterone was, with minor modifications, that used by Sayers(14) in similar studies with other adrenal steroids. Male rats weighing 100-125 g were exposed to a temperature of 5-8° C for 30 minutes. Their adrenals were then removed for ascorbic acid determinations under Nembutal anesthesia. It is known from Sayers' work that in response to such a stress, the adrenal ascorbic acid will fall unless an adrenal steroid is given which will block the stress-induced release of ACTH. The steroids tested were given at a concentration of 200 μg/ml in purified sesame oil (aldosterone may be unstable in USP sesame oil). To achieve solubility of cortisone, 15% benzyl benzoate was added to the oil. The test substances were injected subcutaneously at 2 sites 60 minutes before exposure to cold. All controls received injections of oil; some controls were exposed to cold and others were not. Aldosterone was compared with the free alcohols of cortisone and desoxycorticosterone. The small amounts of aldosterone currently available precluded the use of large numbers of animals or more than 2-dose levels. For that reason, precise relative potency calculations were not attempted.

Results. Results are summarized in Table I. They show that aldosterone had the ability, particularly at a 100 μg dose level, to inhibit the anticipated decrease of adrenal ascorbic acid following stress. Presumably this means that it inhibited the release of ACTH. The potency of aldosterone in this respect lay between that of desoxycorticosterone and cortisone.

Potency ratio calculations based on a 5-dose variance analysis (Bliss (15)) using animals observed simultaneously (Table I) indicated that aldosterone had approximately 38% of the activity of cortisone. The observations on desoxycorticosterone were not made simultaneously with aldosterone, but suggest that aldosterone is in the order of 8 times as active as desoxycorticosterone.

Discussion. These results demonstrate that in the rat aldosterone can antagonize a stress-invoked release of pituitary ACTH. The potency of the compound in this respect, about one-third that of cortisone, closely approximates the "glucocorticoid" activity of aldosterone in other tests such as eosinophil depletion(11,13) and liver glycogen deposition(12). In the less specific cold stress test, the activity of aldosterone equals or exceeds that of cortisone(11) and in affecting electrolyte metabolism and maintaining life it is more potent than any known steroid.

The problem of the physiological relation of aldosterone to pituitary ACTH remains, however, an open question. The doses used in the tests described here may be unphysiological.

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* The aldosterone used here was supplied by Dr. A. Wettstein and his associates, Ciba Ltd., Basle.

TABLE I. Effect of Aldosterone and Other Steroids on Adrenal Ascorbic Acid Content of Rats Exposed to Cold.

	Mean values of all observations		Single experiment–all groups observed simultaneously	
Treatment	No. rats	Ascorbic acid, mg/100 g adrenal ± S.E.	No. rats	Ascorbic acid, mg/100 g adrenal
Control, oil (room temp.)	15	351 ± 21.4		
" " (cold)	39	217 ± 7.1	5	198
Cortisone, 100 μg	34	323 ± 7.5	5	339
50	15	271 ± 10.7	5	305
25	10	249 ± 15.7	5	238
Aldosterone, 100 μg	13	298 ± 11.9	5	276
50	5	218	5	218
Desoxycorticosterone, 800 μg	5	297	Calculated potency:	
400	5	234	Aldosterone = 38% of cortisone	
200	5	234	Confidence limits (P = 0.05):	
100	5	228	26-71%	

Simpson and Tait(5) have estimated recently that the circulating blood level of aldosterone in man is in the order of 0.08 μg/100 ml; such amounts may be inadequate to depress ACTH secretion. Nevertheless, the fact that aldosterone in adequate dosage can exert a wide gamut of cortisone-like effects, including ACTH-suppression, is one of theoretical and potential practical importance.

Summary. Crystalline aldosterone suppressed the release of pituitary ACTH in animals exposed to the stress of cold as judged by adrenal ascorbic acid determinations. Its activity in this respect approximated one-third that of cortisone and eight times that of desoxycorticosterone.

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Passive Anaphylaxis in the Dog. A Quantitative Study. (21316)

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Quantitative studies of passive anaphylaxis have been reported for the guinea pig(1) and the mouse(2). Since no such data for the dog were available, the following experiments were designed to obtain information regarding the sensitivity of this animal to passive transfer of antibody of several concentrations.

It was found that the dog could be sensitized to crystallized bovine serum albumin (Armour Laboratories) through administration of specific antiserum developed in rabbits and representing a total antibody nitrogen content of 21 mg. Fatal anaphylaxis followed intravenous introduction of various amounts of antigen after latent periods of 3¾ to 36 hours.

Materials and methods. Antiserum. The 5 lots of antisera used represented pools of sera obtained from rabbits which had been immunized with crystallized bovine serum albumin in Freund's adjuvants(3) employing 2 parts aqueous antigen, one part Falba and 2 parts Bayol F_3. Rabbits were given repeated subcutaneous injections of these materials but only emulsions prepared for the first 2 doses contained killed and dried *Mycobacterium tuberculosis** in the proportion of 2 mg/ml of Bayol. Animals were bled from the heart after precipitins of suitable titer had been demonstrated. Sera were inactivated at 56° C for 30 minutes and the antibody nitrogen content of pools determined by means of quantitative precipitin procedure(4). Serum pools containing antibody nitrogen of 1.5-2.5 mg per ml were used for sensitization. *Antigen.* The nitrogen content of aqueous solutions of bovine serum albumin was determined by the Kjeldahl method(4). Healthy mongrel dogs weighing 7-9 kg were used. No anesthetic was administered. Antiserum was given intravenously into the jugular or femoral veins and antigen injected by way of femoral vein. Animals were not fed for 24 hours previous to administration of the challenging dose of antigen.

TABLE I. Anaphylaxis in Dogs as Related to the Sensitizing and Shocking Doses and to the Latent Period.

Sensitizing dose, mg antibody N	Latent period, hr	Challenging dose, mg antigen N	Died	Time, min.
21 (2)*	36	17.3	2	150
21 (2)	”	5.4	1	131
21 (1)	”	4.33	0	
16.8 (2)	”	13.0	0	
10.5 (2)	”	8.66	0	
21 (2)	24	17.3	2	84
21 (2)	”	10.88	0	
21 (2)	”	5.4	1	12
16.8 (1)	”	13.0	0	
13.65 (1)	”	10.88	0	
21 (2)	12	17.3	1	45
21 (2)	”	10.88	1	36
21 (4)	”	5.4	0	
21 (2)	6	17.3	1	22
21 (3)	”	5.4	3	15 4 27
10.5 (1)	”	5.4	0	
21 (1)	3¾	5.4	1	21

* No. of dogs in parentheses.

3 additional dogs received 17.3 mg antigen N with no reaction.

Results. The results of these experiments on passive anaphylaxis in dogs using several quantities of antiserum and antigen and with varying latent periods, are presented in Table I. The time interval between the administration of the shocking dose and death is recorded for the 13 instances of fatal anaphylaxis. As shown in Table I, 25 of the 32 dogs received a sensitizing dose of 21 mg of antibody nitrogen contained in 10-15 ml of serum. We postulated that the dog, because of lessened sensitivity, might require 20 times the guinea pig dose(1) per kg for passive sensitization and this amount proved adequate.

The challenging dose of antigen was based on the amount required for complete inhibition *in vitro* with serum of Pool 1. In preliminary experiments, 17.3 mg of antigen nitrogen produced fatal anaphylaxis in dogs sensitized with 21 mg of antibody nitrogen and this result was confirmed with other animals. The 17.3 mg of antigen represented 12 times the amount of bovine serum albumin required for maximum precipitation of antibody in the test tube. This relationship is consistent with the results of Kabat and Landow(1) with ovalbumin, and those of Kabat, Coffin, and Smith(5) utilizing tobacco mosaic virus in guinea pigs.

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* Kindly supplied by Dr. Jules Freund, Public Health Research Institute, New York City.

Four of the 25 dogs sensitized with 21 mg of antibody nitrogen received 17.3 mg antigen nitrogen after latent periods of 36 and 24 hours and died in acute anaphylactic shock. However, when this same dose of antigen was given to 2 dogs after 12 hours and to 2 dogs after 6 hours, only one of each pair exhibited fatal anaphylaxis.

Decrease of the challenging dose produced inconsistent results. 10.88 mg of bovine serum albumin nitrogen failed to elicit death in 2 dogs, when given 24 hours after injection of 21 mg of antibody nitrogen, while one of 2 dogs which received the same dose of antigen after a latent period of 12 hours died in anaphylactic shock. Antigen doses of 5.4 mg of nitrogen produced death in one animal after 24 hours, in 3 dogs after a latent period of 6 hours, and in one after a latent period of 3 hours and 45 minutes. Table I also shows that the interval between the shocking dose and death varied from 4 minutes to 2 hours and 30 minutes. Fatal anaphylaxis was not produced when 7 dogs were sensitized with amounts of antibody varying from 10.5-16.8 mg and subsequently challenged with 4.33-13.0 mg of antigen nitrogen. These amounts of antigen would have inhibited *in vitro* precipitation with the above doses of antibody.

Only one of the 5 pools of antisera produced toxic reactions. Two animals which received this serum developed anaphylactoid reactions with capillary hemorrhage and purpura but recovered. They are not included in Table I. The antigen alone produced no reactions. Three dogs given 17.3 mg of antigen nitrogen showed no ill effects.

The criterion of desensitization was fulfilled by giving one dog a dose of 21 mg of antibody nitrogen followed after 36 hours by 5.4 mg of antigen nitrogen which produced anaphylactic symptoms but with eventual recovery. Two days later the animal was given 17.3 mg of antigen nitrogen and at this time showed no evidence of anaphylaxis.

An immediate fall in blood pressure, ranging from 80-120 mm mercury was the one symptom characteristic of all dogs exhibiting the anaphylactic phenomenon. Other symptoms in the order of appearance were dilatation of pupils, slow, deep, labored respiration, marked prostration, hypertonous, defecation, vomiting, extreme restlessness, salivation, stridor, urination.

Representative sera from dogs in the 6, 12 and 24 hour latent groups were tested for residual rabbit serum but in no instance was such demonstrated by routine precipitin reactions.

Summary. (1) Dogs have been passively sensitized by administration of antiserum to bovine serum albumin developed in the rabbit. 21 mg of antibody nitrogen sensitized each of 25 dogs. All manifested symptoms of anaphylaxis and 13 of these died; 6 (of 8) following a dose of 17.3 mg of antigen nitrogen, 1 (of 4) after 10.88 mg, 6 (of 12) after 5.4 mg, and 1 following a dose of 4.33 mg of bovine serum albumin nitrogen. (2) Latent periods varied from 3¾ to 36 hours. No correlation could be shown between latent period and shocking dose. (3) Sensitizing doses of 10.5-16.8 mg antibody nitrogen followed by antigen in amounts of 4.33-13.0 mg of nitrogen produced symptoms of anaphylaxis in 7 dogs but all recovered. (4) An immediate and rapid fall in blood pressure was the one symptom exhibited by all 32 animals.

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Isolation of a Non-Neurotropic Variant of Type I Poliomyelitis Virus. (21317)

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It is the belief of many investigators working with poliomyelitis that effective prophylaxis against this disease will become possible when highly antigenic, avirulent, or non-neurotropic variants of the virus become available for vaccination purposes. Variants of viruses may appear spontaneously or may be separated out experimentally and "fixed" by various manipulations, including serial passage in a different host, by passage through different routes of tissues in the same host, or by continuous *in vitro* cultivation. By such means, variants of poliomyelitis viruses have not only been shown to exist but, as laboratory technics improve, the isolation of variants exhibiting specifically selected characteristics is becoming more readily accomplished. Theiler(1) demonstrated that the Type II Lansing strain of poliomyelitis virus lost most of its virulence for monkeys after repeated passage in mice. Koprowski *et al.*(2) and Moyer *et al.*(3) demonstrated a similar effect after passing the virus through rodents including mice. Enders and his associates(4) were the first to show that poliomyelitis virus lost most of its pathogenicity for monkeys after serial passage in extraneural human tissue culture. Li and Schaeffer(5) reported that the poliomyelitis virus, Type I Mahoney strain, lost its pathogenic properties for the monkey by the intracerebral route after serial passages in monkey testicular tissue cultures, while gaining at the same time the new property of mouse pathogenicity via the intraspinal route. Li and Schaeffer(6) also reported that the mouse-adapted Leon virus lost all of its mouse and most of its monkey pathogenicity after serial passages in monkey testicular tissue cultures. Sabin *et al.*(7) recently "converted" highly virulent strains of the 3 immunogenic types of poliomyelitis virus into avirulent variants by serial passage at 24-hour intervals of large inocula of virus in monkey kidney tissue culture and purification of such culture fluids by the "terminal dilution" technic. Melnick(8) also obtained avirulent variants of the poliomyelitis viruses in tissue culture. Most recently, Koprowski *et al.*(9) were able to obtain a rodent adapted mutant of Type I poliomyelitis virus from a mixture of two strains of virus.

The present communication describes the selection of another interesting variant of Type I poliomyelitis virus strain derived from a line which was previously reported(5) to have given rise to a mouse-adapted strain. This variant, after further consecutive passages in tissue culture (monkey testis and kidney), was subjected to alternate intracutaneous inoculation in monkey skin and monkey kidney tissue culture. After a number of such alternate passages the mouse pathogenicity, which was only recently gained, again disappeared. Pathogenicity for the monkey could not be demonstrated even by direct intraspinal inoculation but the immunogenic characteristics of the variant remained unaltered.

Materials and methods. Virus. A variant of the Mahoney strain of Type I poliomyelitis virus adapted to mice by the intraspinal route as previously reported(5) was employed in this study. This substrain will be designated as LS-a. While this variant exhibited certain differential characteristics, it soon became obvious that suspensions of the variant virus did not contain single homogeneous virus particles but rather consisted of a mixture of a number of different variants. Supporting evidence for this belief is to be found in the data presented below and also the reports of Stanley *et al.*(10) and Krech(11), who received subpassages of the LS-a strain which had not been readily infective to mice by the intracerebral route in our laboratory. With further serial intracerebral passage in mice, these authors each obtained a variant of this virus which easily infects mice by this route. Subsequently, a similar variant was obtained in our laboratory by Dr. A. B. Eschenbrenner(12). *Tissue culture.* At the beginning of the serial tissue culture passages, monkey testicular tissue was employed and these were prepared in the manner described previously(13). Later monkey kidney cell suspensions were substituted. These were prepared by the method described by Youngner(14) with slight modification. Trypsinized monkey kidney cell suspensions were suspended in a nutrient of 30% horse serum, 10% chicken embryo extract prepared according to the method of Bryant *et al.*(15), and 60% Hanks' solution. The cell suspensions were distributed into tubes in 0.5 ml amounts. The tubes were incubated for 6-8 days at 35° C; the fluid was removed and the cells were washed with neutralized Hanks' and the fluid replaced with 1.5 ml of Medium E(13). Such tubes were used for virus inoculation or neutralization tests immediately or within a week. All tubes were incubated in stationary position. For virus passage in tissue culture usually 0.2 ml of the undiluted culture fluid was added as the inoculum and the tubes further incubated for 3 days for harvesting, when cellular degeneration was almost complete. For tissue culture virus titration, 0.1 ml amounts of the 10-fold virus dilutions were inoculated into each of 2 tubes containing 1.5 ml of medium, and the tubes were further incubated for 7-8 days for final readings. Presence of virus was based on complete or definite cellular reaction within 8 days. For animal pathogenicity tests or neutralization tests, a "terminal dilution" tissue culture subpassage was made and the subpassage material employed in such tests. *Mice.* Four- to 5-week-old CFW white mice were used. They were usually inoculated by the intraspinal technic which has been described previously(16). *Monkeys.* Rhesus monkeys weighing 2-5 lb were used for the first 2 virus passages, and Cynomolgus monkeys weighing 2-4 lb were used for the later passages. The skin of the abdomen was shaved without application of any antiseptics. The virus inoculum was introduced into the skin with a 1 ml syringe and #27 gauge ¼-inch needle. The inoculum of undiluted tissue culture fluid, with or without adjuvants, was introduced intradermally in a series of 10 single blebs in 0.1 ml amounts placed adjacent to each other. In this manner, a total amount of 1 ml was injected in a cluster of individual intracutaneous inoculations within an area of about 3 x 3 cm. Schick toxin (diphtheria toxin for Schick test, diluted, Wyeth Laboratories, Mariatta, Pa.), or diphtheria-tetanus-pertussis combined vaccine (Tri-immunol, Lederle Laboratories) was used as "adjuvant" in some instances. When used, the "adjuvant" was diluted in half with buffered saline and mixed with the virus-containing tissue culture fluid in equal amounts. From 3-6 days after inoculation, the inoculated area was observed for reactions, then washed with soap followed by sterile water and alcohol and, lastly, cleaned with cotton swabs soaked in saline containing 1000 units penicillin and 500 μg streptomycin per ml. The entire inoculated area of the skin was then removed with a pair of scissors, washed again with saline and with antibiotics, ground thoroughly with alundum in a mortar and a 10% suspension was prepared in buffered saline. After centrifugation, antibiotics in the stated concentrations were added to the supernate and 0.2 ml was inoculated to each of 3 or 4 tissue culture tubes. When complete degeneration of the cells had occurred, usually within 3-7 days, the undiluted fluid was harvested and reinoculated to monkey skin, with or without "adjuvants" as described above. For intraspinal inoculation the monkey* was held on the abdomen with the back arched. The intravertebral spaces were counted upwards from the first space below the level of the iliac crest, upwards until the fifth intravertebral space was located and inoculation was made there, with a ¼ ml syringe and a 1-inch #22 or #23 gauge needle, directly into the spinal cord. Monkeys were observed usually for 21 days after inoculation for any signs of paralysis or other symptoms. Paralyzed monkeys were sacrificed and sections from the spinal cord and the brain stem were examined for histopathologic changes. Monkeys which were not paralyzed were similarly examined within or after the observation period.

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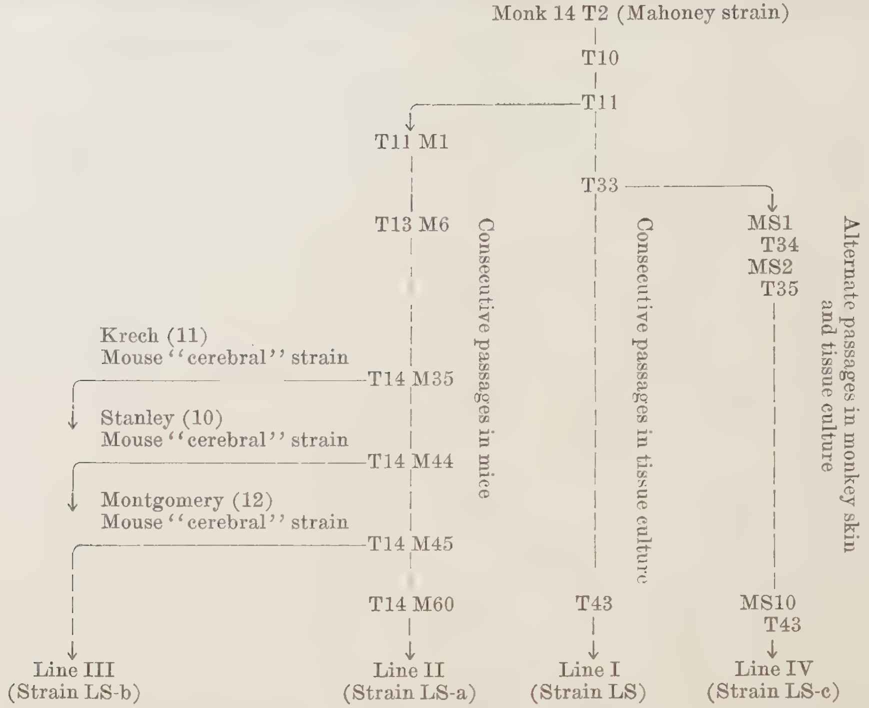
* For this technic and its application to virulence tests of poliomyelitis virus in monkeys, the authors are indebted to Dr. A. B. Sabin, who kindly supplied the results of his unpublished experiments.

CHART I. Lines of Passage of Poliomyelitis Type I Virus Resulting in Variants.



T = Tissue culture. M = Mouse. MS = Monkey skin.

Results. The Mahoney strain of Type I virus when received in this laboratory from Dr. Jonas E. Salk had had 14 monkey passages and 2 tissue culture passages. After 9 additional tissue culture passages, it became apparent that a variant of the virus had appeared. This was recognized by the acquired ability of the virus to produce infection by direct inoculation into the spinal cord of rodents. At the same time, this virus also lost its pathogenicity for monkeys by the intracerebral route, but it was found subsequently that occasional monkeys could be infected by direct intraspinal inoculation of this variant.

With further subpassages of this virus, under conditions described below, there were established 4 lines of the virus. Three of these appear to be distinct variants which exhibit characteristic host range differences but which have similar antigenic properties. These passage lines are presented graphically in Chart I.

Line I is derived from the original Mahoney strain and has been passed consecutively in tissue cultures. The first 15 passages were in monkey testicular tissue and the subsequent 29 passages in monkey kidney cell preparations. This variant is designated as the LS strain.

Line II is a derivative of Line I and was recognized as a *spinal variant* because it could infect mice readily only by the intraspinal route. This variant which is designated as LS-a has retained but low virulence for the monkey which is demonstrable only by direct intraspinal inoculation.

Line III, a derivative of Line II, following consecutive mouse intraspinal passages gave rise to a variant of the virus capable of infecting mice by the intracerebral route. Although there were indications in earlier passages that some mice would succumb following intracranial infection, it was not until more serious efforts were made with material from the 45th intraspinal mouse passage that a "cerebral" strain was derived(12). This strain is designated as LS-b. Similar strains had been obtained previously by Krech(11) from our 35th

TABLE I. Reactions of Variants of Poliomyelitis Virus, Type I, Derived from Mahoney Strain.

	Host range				
	Tissue culture (monkey kidney)	Monkey		Mouse	
Variant		Intracerebral	Intraspinal	Intracerebral	Intraspinal
Mahoney	+	+	+	—	—
LS	+	—	+	—	+
LS-a	+	—	+	—	+
-b	+	—	+	+	+
-c	+	—	—	—	—

passage and by Stanley(10) from our 44th passage.

Line IV was derived from the 33rd consecutive tissue culture passage of Line I. This was inoculated intracutaneously into a monkey and thereafter it was carried to tissue culture and monkey skin alternately. It is currently in the 10th monkey skin and 43rd tissue culture passage. This strain, designated as LS-c, continues to grow well in tissue cultures but has become avirulent for mice and monkeys by all routes since the 7th monkey skin passage.

Table I presents the basis for differentiating the derived variants or mutants. The variation of host reactions shown have been determined by repeated tests with confirmatory results. The parent Mahoney strain has tissue culture and monkey pathogenicity but lacks mouse pathogenicity. Strains LS and LS-a, which appear to be identical, have lost monkey intracerebral pathogenicity but gained mouse intraspinal pathogenicity. Strain LS-b is similar to LS and LS-a but in addition has mouse intracerebral pathogenicity. Strain LS-c, the most interesting of these derived strains, may be, or is the forerunner of, a much-sought-for avirulent strain. Further details concerning the monkey pathogenicity of these strains are shown in Table II. In one series of 18 monkeys which were inoculated intraspinally with avirulent strain LS-c, all re-

TABLE II. Comparison of Tissue Culture Titer, Mouse and Monkey Pathogenicity of Three Virus Variants.

Test	Line II (strain LS-a)	Line I (strain LS)	Line IV (strain LS-c)
Tissue culture titer	10^{-6} to 10^{-7}	10^{-6} to 10^{-7}	10^{-6} to 10^{-7}
Intraspinal infectivity in mice	Titer $10^{-2.5}$ to $10^{-3.0}$	Titer $10^{-2.0}$ to $10^{-2.5}$	Negative in several tests totalling over 200 mice*
Intracerebral infectivity in monkeys	M7, M8, M9† 0/6§	T21‡ 0/6	MS8 T41‖ 0/6
Intraspinal infectivity in monkeys	M56¶ 10^{-1} 10^{-2} 0/3 2/3	T21 T33 T43 4/6 1/6 1/6	Series 1 MS8 T41 MS8 T41 MS9 T42 0/6 0/6 0/6 Series 2 MS10 T43 after 5 skin grafts on egg passages Undil. 10^{-1} 10^{-2} 0/3 0/3 0/3

* Virus may survive in some cords tested 3, 4, and 7 days following intraspinal inoculation but neither symptoms nor histologic evidence of infection were observed.

† Cords of mouse passages 7, 8, 9 tested as 10% suspension. Passage M23 also failed to infect 6 monkeys.

‡ Tissue culture passage 21 tested.

§ No. of monkeys paralyzed or dead over monkeys inoculated.

‖ MS8 T41 = Alternate monkey skin passage 8 and tissue culture passage 41 tested.

¶ Mouse cord passage 56 tested, 10% (10^{-1}) cord suspension in 3 monkeys and 10-fold dilution (10^{-2}) of suspension in another 3 monkeys. Note more dilute virus infected while undiluted 10% suspension did not.

TABLE III. Gain and Loss of Mouse "Spinal" Pathogenicity with Passage of Poliomyelitis Virus, Type I.

Passage	Tissue culture titer	Mouse intraspinal infectivity	
		Undiluted	Titer
MK14 T2 (Mahoney strain)		Negative	Negative
T11		9/35	<1 LD_{50}
T21		7/7	$10^{-2.5}$
T33*	10^{-6}	30/30	$10^{-2.2}$
MS1† T34			
MS2 T35		6/7	$10^{-2.2}$
MS7 T40		0/6	
MS8 T41	10^{-7}	0/40‡	
MS9 T42	10^{-7}	0/40	
MS10 T43	10^{-7}		
T43	10^{-7}	10/10	$10^{-2.5}$
(Strain LS) (Strain LS-c)			

* T33 = Tissue culture passage No. 33.

† MS1 = Monkey skin passage No. 1.

‡ 10 mice each also received material from this passage diluted 10^{-1} and 10^{-2} with negative results.

mained asymptomatic. These animals were sacrificed after varying observation periods from 21-35 days and their CNS tissues examined for histopathologic lesions. All were negative with the exception of one monkey which showed some questionable lesions confined to the site of inoculation in the spinal cord. These lesions were not entirely typical of poliomyelitis and could be attributed to inoculation trauma with superimposed bacterial infection.

Table III illustrates the chronologic gain and loss of mouse pathogenicity with reference to strain LS-a and shows that despite 43 consecutive tissue culture passages, strain LS has retained its virulence for the mouse. The tissue culture characteristics of all of the variants have remained essentially unaltered and periodic neutralization tests conducted with these strains have shown them to react specifically as Type I poliomyelitis virus. The LS-c strain is being carried further in alternate monkey skin and tissue culture passages in attempt to establish a dermotropic variant and to remove, if possible, by this means, any remaining neurotropic virus particles.

To further augment this possible dermal tropism, passage MS10 T43 of the LS-c strain received 5 consecutive passages to monkey skin grafted onto the chorioallantoic membrane of chick embryos(17). Following this, it was transferred to tissue culture and the tissue cultured passage fluid was diluted serially in 3 ten-fold steps and each dilution inoculated into the spinal cords of 3 monkeys (series 2, Table II). None of these monkeys developed any symptoms and since infection in the monkey by spinal route usually occurs within 3-7 days, the monkeys were sacrificed on the ninth day for histopathologic examination. No lesions were found in this group of monkeys.

Preliminary experiments just completed and others still in progress indicate that current passages of the LS-c strain may multiply, or at least survive at the site of intracutaneous inoculation in the monkey. Such inoculations of this virus induce immunity, as evidenced by the appearance of antibodies and resistance to paralytic infection following challenge with the highly virulent Mahoney strain. There is also suggestive evidence that when the virus is inoculated in combination with "adjuvants" such as diphtheria-pertussis-tetanus vaccine there is an increased local virus activity with enhanced immune response.

Summary. Following the adaptation of Type I, Mahoney strain, poliomyelitis virus to mice, further passages in tissue cultures, mice, and alternate monkey skin to tissue cultures resulted in the establishment of 4 passage lines of virus, 3 of which appear to be distinct variants. These strains have been designated as LS, LS-a, LS-b and LS-c and are differentiated on the basis of their range of host reactions. LS and LS-a react similarly and are so-called mouse and monkey spinal variants. LS-b is a mouse cerebral strain and LS-c is a non-neurotropic strain which is avirulent for both mice and monkeys by all routes. These variants have unaltered antigenic and tissue culture characteristics. Preliminary experiments with the LS-c strain have shown it to be capable of inducing good antibody titers and a high degree of resistance to virulent virus challenge in monkeys. There is suggestive evidence that continued skin passages of the LS-c strain may yield a dermal poliomyelitis virus variant.

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Multiplication of Poliomyelitis Virus in Monkey Tissues Grafted onto Chorioallantois of Chick Embryos. (21318)

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The propagation and cytopathogenicity of the poliomyelitis viruses in tissue culture and their adaptation to mice have greatly enhanced laboratory investigations of these viruses. Both the *in vitro* and *in vivo* methods are now widely used for fundamental studies and their practical application to epidemiology and prophylaxis is currently in progress. However, for fundamental studies for the virus-host relationship, further search for investigative tools is still necessary. Roca-Garcia *et al.*(1) and Cabasso *et al.*(2,3) recently reported successful propagation of Type II poliomyelitis virus in the embryonated chicken egg. Although numerous attempts have been made in this direction with the other two types of virus, the results thus far have been negative. It was thought, therefore, that isolated but organized fragments of susceptible host tissue grafted onto the embryonated egg membrane might offer an intermediate step in the adaptation of poliomyelitis virus to the chick embryo. Goodpasture, *et al.*(4) first described a technic for grafting human skin onto the embryonated egg membrane. Since then various workers have implanted a variety of tissues of human or animal origin to this membrane, studied the growth of the implanted tissue and the reaction of the host, and utilized the technic for the cultivation and investigation of viruses and rickettsiae, including hog cholera, vaccina, herpes simplex, herpes zoster, and fowlpox viruses(5-13).

The present report describes the successful propagation of poliomyelitis virus of all 3 immunologic types in monkey tissues implanted onto the chorioallantoic membrane (CAM) of embryonated chicken eggs. Although the original goal of adapting the virus to chick embryos has not, so far, been reached; no "adaptation" was found necessary for multiplication of the virus on the grafted tissues by the technic described.

Materials and methods. Virus. A number of substrains of modified poliomyelitis virus, Type I, Mahoney strain(14) were used for this study. The details concerning some of the characteristics of these substrains have been reported(15). Among the various strains was included mouse-adapted Type I virus which was sent to Dr. N. F. Stanley in Australia, after 44 mouse intraspinal passages. The virus was adapted there to mice intracerebrally(16). After 12 intracerebral passages in mice the virus was sent back to this laboratory where it received 5 additional tissue culture passages. This strain has been designated the LS-b strain(15). Type II MEF_1 strain, and mouse-adapted Type III Leon strain(17) were also used in these studies. These viruses were further propagated in monkey kidney tissue culture for several passages and the undiluted tissue culture fluid was then used to initiate the first CAM-kidney tissue graft passage. *Tissue grafting.* For the preparation of kidney tissue, monkeys were sacrificed and their kidneys excised. The cortex of the kidney was cut out and minced with scissors into fragments of about 2 mm in diameter. The minced tissue was then washed in neutralized Hanks' solution about 10 times until the supernate became clear. The tissue was then suspended in medium consisting of 30% monkey serum, 10% chick embryo extract and 60% Hanks' solution. The pH was adjusted to 7.5 and 50 μg of streptomycin and 100 units of penicillin per ml were added. White Leghorn embryonated eggs incubated for 6 or 7 days were prepared with a false air sac and a window about 8 mm in diameter was made on the side of the egg overlaying the artificial air sac. The kidney tissue was then dropped onto the egg membrane with a capillary pipette. About 10 pieces of tissue were implanted on each egg membrane, the window sealed with sterile lens paper and paraffin, then the eggs were incubated for 24 hours before virus inoculation. When monkey skin was used for grafting, instead of monkey kidney, the skin was taken from the abdomen after it was cleansed with soap, water, alcohol, and saline containing 500 γ of streptomycin and 1000 units of penicillin per ml. Most of the fat was removed and the skin was cut into small pieces and grafted in the same manner as the kidney tissue. *Virus Inoculation, Harvesting, and Titration.* The virus contained in inocula of 0.5 ml was dropped onto the grafted membrane with a syringe and a 22 gauge needle through a hole made in the lens paper and the hole again sealed with paraffin. The eggs were further incubated, usually for 5 days, before virus harvesting. Then the shell was swabbed with tincture of iodine and an opening was made over the artificial air sac through which grafted tissue could be carefully examined. Eggs without successful graft tissue were discarded. From the others the grafted tissue with some of the attached egg membrane was first removed. Then the allantoic fluid, the embryo, and the remainder of the CAM were harvested and the embryos,

TABLE I. Evidence of Virus Multiplication in Monkey Tissues Grafted on CAM.

Series	Virus Type	Virus Strain	Monkey tissue grafted	Inoculum Passage No.	Inoculum Titer	Titer of harvested grafts Passage No.	Titer of harvested grafts Titer
I	I	LS-b	kidney	E8	10^{-2}	E 9	10^{-6}
				E9	10^{-3}	E10	10^{-7}
II	II	MEF$_1$	”	E1	10^{-2}	E 2	10^{-5}
				E4	10^{-4}	E 5	”
III	III	Leon LH	”	E1	10^{-3}	E 2	”
				E4	10^{-3}	E 5	”
IV	I	LS-c	skin	E6	10^{-2}	E 7	10^{-4}

membranes, and kidney fragments were ground separately in a mortar and suspended in neutralized Hanks' solution. Usually a 10% suspension of the kidney fragments and the membrane and a 20% suspension of the embryo were made. The fluids were centrifuged and 200 units of penicillin and 100 μg of streptomycin per ml were added to the supernates which were used undiluted for further passages. To titrate for virus content, 10-fold serial dilutions in Hanks' solution were made and 0.1 ml of each dilution was inoculated to each of 2 monkey kidney tissue culture tubes containing 1.5 ml of medium and incubated at 35°C. Evidence of virus activity in the lower dilutions usually appeared in the cultures on the first or second day after inoculation but the titer was finally determined at the end of 7 or 8 days of observation. The kidney fragments were also titrated at the same time in mice by the intraspinal route (18).

Results. Four different series of passages of poliomyelitis virus in embryonated eggs grafted with monkey tissue were carried out.

Series 1. This series was initiated with Type I, LS-b strain(15) which had 56 passages in mice and 19 passages in tissue culture. The virus (contained in tissue culture fluid) was inoculated onto the CAM of embryonated eggs which had been grafted with monkey kidney tissue 24 hours previously. After 5 days of incubation the CAM and embryos were harvested and a 66% suspension was made in their own allantoic fluid. Ungrafted 7-day-old embryonated eggs were inoculated with 1.0 ml of this suspension into the yolk sac. In 4 consecutive passages thus carried on chick embryos without implants, the virus content decreased steadily and by the 5th passage it was completely lost (Table II, passage E1 to E5a). A new start was made using the embryo membrane suspension from the 3rd passage as inoculum and kidney tissue grafted on CAM as the host (Table II, passage E4). Subsequently, the virus was carried in 12 passages consecutively in such grafts, except for passage E10 which was an ungrafted embryonated egg passage. As seen in Table II, in occasional passages the inoculum consisted of CAM, allantoic fluid, or embryo, instead of kidney graft suspensions.

Series 2 and 3. Type II, MEF$_1$ strain and Type III, mouse-adapted Leon strain, were also successfully grown in the grafted kidney tissue on the embryonated egg membrane for 5 consecutive passages. Multiplication was demonstrated in both instances by significant increases in virus titer on the grafts. For example, the inoculum (CAM suspension) for the second passage of Series 2 had a tissue culture titer of 10^{-2}. When this was grown on grafted tissue and harvested, the material had a tissue culture titer of 10^{-5} (Table I, Series 2). The virus continued to multiply in this fashion in successive passages.

Series 4. In this series monkey skin instead of monkey kidney tissue was grafted onto the embryonated egg membrane. Infection was initiated with the tissue culture fluid of Type I virus, non-neurotropic substrain LS-c, which previously had had 43 tissue culture passages and 10 passages by intracutaneous inoculation of monkeys(15). Seven consecutive passages were carried on embryonated eggs grafted with monkey skin. Although the in-

TABLE II. Propagation of Poliomyelitis Virus, Type I, (Strain LS-b), in 12 Passages in Monkey Kidney Tissue Grafted on CAM of Embryonated Eggs (Series I).

					Titer of harvested grafts in tissue culture:					
Passage No.	Embryonated egg preparation	Site of inoculation-	Inoculum (0.5 ml)	Titer	Kid.	CAM	All. fl.	Emb.	Emb. + CAM + all. fl.	Titer of harvested grafts in mice
E1	Kid. G*	Kid. G	T.C. fl.	10^{-7}					+	
E2	U	Yolk sac	Emb. + CAM + all. fl.						+	
E3	U	”	”						+	
E4a	U	”	”						+	
E5a	U	”	”						—	
E4	Kid. G	Kid. G	”		10^{-6}		10^{-2}			$10^{-1.5}$
E5	”	”	Emb.	10^{-2}	”		”	10^{-2}		10^{-3}
E6	”	”	”	10^{-2}	”		10^{-3}			$10^{-2.7}$
E7	”	”	Kid. G	10^{-6}	10^{-7}	10^{-4}	10^{-2}	10^{-3}		$10^{-2.5}$
E8	”	”	”	10^{-5}	10^{-6}	10^{-2}	10^{-1}	10^{-2}		$10^{-3.0}$
E9	”	”	Emb.	10^{-2}	”	10^{-3}	10^{-4}	”		$10^{-2.5}$
E10	U	Yolk sac	Kid. G	10^{-6}			10^{-1}	”		
E10a	Kid. G	Kid. G	CAM		10^{-7}		10^{-3}	”		10^{-3}
E11	”	”	All. fl.							
E11a	”	”	Emb.	10^{-2}	10^{-6}					$10^{-2.2}$
E12	”	”	Kid. G	$>10^{-3}$	”	10^{-3}	10^{-2}			$10^{-2.6}$

* G = grafted; U = ungrafted.

oculum usually contained 5 x 10^2 tissue culture infecting doses, the average tissue culture titer of virus harvested from skin grafts in each passage was about 10^{-4} to 10^{-5}. Similar virus titers were observed in harvests taken after 5 and 7 days' incubation but there was considerable decrease in virus content when harvesting was delayed until the ninth day. Since inocula containing virus in the vicinity of 10^{-2} tissue culture infecting doses failed to yield significant amounts of virus when introduced into the CAM or yolk sac of ungrafted embryos but resulted in titers of 10^{-6} or 10^{-7} in each successive tissue graft on CAM passage, there is ample evidence that the virus multiplies in the latter preparation. Neutralization tests performed with standard poliomyelitis immune sera against virus selected from certain egg passages indicate that the immunologic specificity of the virus was not altered by such propagation.

Histologic findings. Some of the harvested tissue implants were examined fresh under a dissecting microscope, while others were fixed in 10% formalin, sectioned, and stained for histologic study. As described previously by others(10,13), in successful "takes" the implants become engulfed by the chorioallantoic membrane. Proliferation of connective tissue into the tissue spaces and invading chick capillaries, as evidenced by nucleated erythrocytes, were seen penetrating into the grafts and establishing circulation. Varying degrees of organization, absorption, fibrosis, and dissolution were observed but some of the implanted tissue remained viable and the structure was well preserved in many instances.

When virus was added, cytopathogenic effects were frequently not visible and recognition of the specifically reacting cells was difficult. In kidney tissue grafts, degenerative reactions, when seen, were most prominent in the interstitial cells surrounding the tubular islands, but occasionally nonspecific reactions of similar nature were observed in uninfected implants. Further studies concerning the histologic picture in tissues implanted and infected on the CAM are in progress and will be reported later.

Conclusions. These studies have demonstrated that all 3 immunologic types of poliomyelitis virus may be propagated successfully in monkey kidney and skin tissue grafted onto embryonated egg membrane. No preliminary "adaptation" is necessary for virus multiplication in this system. Presence of virus is not limited to the grafted tissue but is found also in moderate concentration in the allantoic fluid, the CAM, and the embryo. However, this is probably due to spillage and dissemination from the grafts where virus growth apparently occurs. More precise knowledge is needed with reference to the exact sites of multiplication and the specific cells participating in this activity.

Further studies are desirable to determine the practical and theoretical applications of this technic and whether simplification and improvements are possible.

Summary. Successful cultivation of all three immunologic types of poliomyelitis virus in monkey kidney and skin tissues grafted onto embryonated egg membrane is reported. In one series Type I virus, LS-b strain, was cultivated for 12 consecutive passages. The virus was found in high titer in the grafted tissue and also disseminated in lower concentration into the allantoic fluid in the chorioallantoic membrane and in the embryo. This technic is being further explored for useful laboratory applications.

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Comparative Study of Effects of Phenothiazine and Iodine on Thyroid Uptake of I^{131}. (21319)

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It has been shown recently(1) that phenothiazine N. F. (green)* inhibited the uptake of radioiodide by the rat thyroid when fed *ad libitum* in the diet. Also, Talmage *et al.*(2,3) reported that a similar inhibition could be produced by the drug when administered orally to such stock animals as burros, sheep, hogs, cattle, and chickens. After using various commercial preparations, including one purified form of the drug, they indicated that the factor which inhibited the uptake of I^{131} was confined to the impurities in the drug. One of these found in most commercial preparations contains organically bound iodine which is incorporated during the use of this element as a catalyst in the manufacturing process. If this iodine were readily dissociable from the organic nucleus to which it is attached, it could explain the inhibitory powers of the drug. For, as Williams *et al.*(4) have shown, inorganic iodide in sufficient amounts will inhibit the uptake of radioiodide by the thyroid.

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* Phenothiazine N. F. (green) is the commercial form of drug which, though conforming with National Formulary Standards, contains significant amounts of impurities which contribute to its green color.

The purpose of the present work was to clarify the role which iodides play in the inhibition of I^{131} uptake by the thyroid which occurs when an animal is dosed with phenothiazine N.F. (green).* Should the iodine be shown to dissociate freely from its organic union when the drug reached the gut, it could be concluded that the inhibition of the uptake of radioiodine was due to prior saturation of the thyroid gland with stable iodide released from the drug. However, if it could be shown that inorganic iodide ions were not immediately available, it might be assumed that the drug contained an active antithyroid agent. The following studies clarify this point and indicate the possible presence of an antithyroid agent in most commercial preparations of phenothiazine.

Materials and methods: Male Sprague-Dawley rats, weighing between 150 and 250 g, maintained on Remington low-iodine test diet (5) and distilled water, were used throughout these experiments. A single stock of phenothiazine N.F. (green)[†] was used as the primary test material. This was analyzed separately by a commercial laboratory[‡] and found to contain 0.4% iodine. Other test materials used included phenothiazine N.F. (yellow),[†] diphenylamine,[†] bromine catalyzed phenothiazine,[†] thionol[§] and phenothiazine sulfoxide.[§] All materials were administered by stomach tube under light ether anesthesia. When insoluble, these were suspended in distilled water with the aid of a wetting agent. Controls given the wetting agent alone, showed no inhibition of radioiodide uptake by the thyroid gland. Carrier-free I^{131} was used for all tests and was administered either orally or subcutaneously as appropriate for the particular experiment. At the conclusion of the experiment, the thyroid glands were removed, placed in small vials and counted directly by means of a well-type (Toroidal) gamma counter.[‖] Paper chromatographic separations were run, using both Whatman's #1 and #4 paper. The solvents used will be described below. The column chromatographic separations utilized both pre-treated alumina and analytical grade silicic acid. Finely divided alumina was pre-treated by washing in hot distilled water until the washings were neutral, and activated by heating to 200° C *in vacuo* for one hour. The silicic acid was activated by pre-washing with two volumes each of the following solvents in sequence: absolute ether, ether and acetone (1:1), ether, and benzene. Comparable results were obtained with both absorbent systems.

Results. Comparison of thyroid uptake produced by phenothiazine N.F. (green): In order to compare the effect of phenothiazine N.F. (green) on the thyroid with that of an excess of free iodide ions, a series of experiments were run in which equimolar amounts of iodide, both as KI and as phenothiazine N.F. (green) were administered in varying degrees to rats. The resultant curves are plotted in Fig. 1. For these experiments both the phenothiazine N.F. (green) and the KI were administered by stomach tube followed immediately by carrier-free radioiodide similarly administered, and the animals were sacrificed 5 hours later. The data are presented as per cent of injected dose of I^{131} taken up per mg of thyroid tissue of the rats plotted against μg of available iodine administered either as KI or as phenothiazine N.F. (green). It can be noted that except for minor differences, the 2 curves are similar. Since the radioiodine was administered with the test materials and the thyroid glands were removed 5 hours later, there is little doubt that if the inhibitory effect of phenothiazine is due to its iodine content, this iodine must become immediately available to the circulation as free iodide ions.

Effect of continuous feeding of phenothiazine N.F. (green) on organ weight: Administration of inorganic iodide is known in some instances to suppress the activity of the thyroid, although it is not considered to be goitro-

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[†] The Dupont Co., La Porte, Texas.

[‡] Huffman Microanalytical Laboratory, Wheatridge, Col.

[§] Synthesized by one of the authors (R.H.B.) at the VAH, Radioisotope Lab., Houston, Texas.

[‖] Manufactured by The Texas Co.

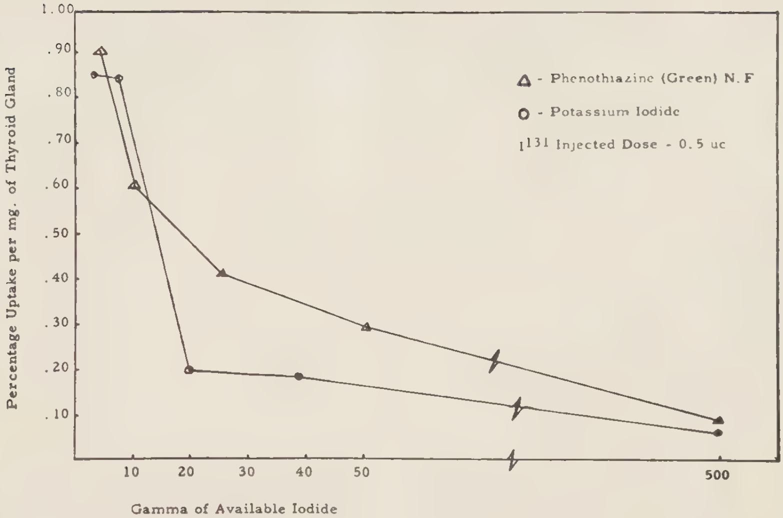

FIG. 1. Depression of I^{131} uptake of thyroid gland. (Each point represents avg of 5 rats.)

genic. This is presumably due to a simultaneous suppression of pituitary TSH and the formation of thyroxin by the thyroid gland (6). To compare the action of phenothiazine N.F. (green) with inorganic iodide in this regard, 16 rats were maintained on stock diet including phenothiazine N.F. (green) .075% by weight, fed *ad libitum* for a period of 14 days. This dose represents a low non-toxic level which elicits a moderate reduction in I^{131} uptake by the thyroid. Total body weights and thyroid, liver, testes, adrenals, kidneys and spleen weights were taken and there was no significant change in the weight of any of the organs, including the thyroid. The thyroid, however, showed approximately a 50% reduction in the uptake of radioiodide. This experiment illustrated again the similarity in action of phenothiazine N.F. (green) to that of inorganic iodide. In the light of the possibility that there exists in the commercial forms of this drug a substance with thyroid function suppressing activity, these data are significant as they indicate that such an inhibitor would not be goitrogenic.

Thyroid uptake inhibition by various chromatographic fractions of phenothiazine N.F. (green) and of related compounds: Column chromatographic separations of commercial phenothiazine were run with the adsorbents described above. The column was developed with solvents of increasing polarity in the following order: benzene, which removed most of the yellow pure phenothiazine and approximately 75% of the originally applied material; benzene-chloroform; chloroform; methanol and chloroform; and methanol. Four fractions, each containing several compounds, were obtained and assayed in rats for their ability to inhibit I^{131} uptake by the thyroid. The data are summarized in Table I. Total iodine analysis‡ indicated that only Fraction IV contained a significant amount of iodine, and that this had increased to 2.04% by weight. This fraction inhibited the radioiodide uptake by the thyroid to a degree similar to that when equimolar amounts of inorganic iodide, as NaI, were administered. For additional comparison, a group of related compounds was assayed in rats for their thyroid-uptake-suppressing ability, the results of which are tabulated in Table II. Particular

TABLE I. Effect on I^{131} Thyroid Uptake by Fractions Eluted from Activated Alumina Chromatographic Column.

Fraction	% thyroid uptake*	Elution solvent
I	26.2	Benzene
II	27.7	$CHCl_3$
III	24.4	$CHCl_3$ - CH_3OH
IV	2.2	CH_3OH
Sodium iodide	2.7	
Controls	20.2	

* Uptake by total thyroid of carrier-free I^{131}.

TABLE II. Summary of Effect of Various Associated and Derived Compounds of Phenothiazine on the % Uptake of I^{131} by Rat Thyroid.

Material	Dose, amt/rat/day	Iodine content	% I^{131} uptake[*]
Low iodine diet controls	—	Low	22.2
Phenothiazine N. F. (green)	10 mg	.40	5.5[‡]
" " (purified)	10	.0[†]	17.8[‡]
" " "	50	.0	14.2[‡]
" " (green) bromine catalyzed	10	.0	21.6
Sodium iodide	100 μg	79.4	2.7[‡]
Diphenylamine (recrystallized)	5 mg	.0	21.4
Phenothiazine, 5 sulfoxide	5	.0	23.2
Phenothiozone (thionol)	5	.0	20.1

[*] Each value represents avg of 3 or more rats.
[†] Less than .002%.
[‡] P values = less than .01.

interest in this connection is the failure of phenothiazine prepared with bromine as a catalyst to inhibit thyroid uptake and the partial but significant suppression of thyroid uptake produced by commercially purified phenothiazine N.F. (yellow), which contains only traces of iodine as a contaminant.

Renal iodide excretion following phenothiazine N.F. (green) and NaI Administration: The inhibitory effects of commercial phenothiazine and of inorganic iodide have been shown above to be parallel. The only exception was the partial inhibition by purified phenothiazine. As has been pointed out, if the inhibition of thyroid uptake of radioiodide by phenothiazine N.F. (green) were due to its iodide content, this must be liberated in the body immediately as an inorganic ion. Such an occurrence should raise the level of the circulating ion and increase its excretion through the kidney. Paper chromatographic analyses of urinary iodides were therefore run to determine the presence of this ion in the urine after the animal had been treated with phenothiazine N.F. (green) and with NaI. In order to decrease the renal excretion of iodides before administration of the test substances, the animals were maintained on Remington low iodine test diet for eight to fourteen days. Test materials were administered by stomach tube, and urine was collected for 24 hours. Three groups of animals receiving either 800 μg equimolar iodide in sodium iodide, phenothiazine N.F. (green), or a combination of both comprised the experimental groups while two groups, one on stock diet and one on Remington low iodine diet, served as controls. Groups 1, 2, 3, and 4 were on Remington low iodine test diet for 14 days. Group 5 received Purina lab. chow exclusively.

In preparation for the chromatogram, each 24-hour urine sample was dried under vacuum at 60° C and brought to a volume of 5 mls with triple distilled water, after which 1 μc of carrier-free I^{131} was added to each sample.[¶] One sample from each test animal was spotted on the same sheet of Whatman #1 paper with a known standard of NaI. The chromatograms were developed in a butanol-acetic acid-water (500:140:40) solvent system, using either a descending or ascending technic. For best separation from the chlorides, the descending technic was used. The iodide spot was detected as a blue spot after spraying with A_gNO_3, washing with dilute HNO_3, drying in the dark, and exposing to ultraviolet radiation. Autoradiograms were made from the chromatographic sheet before the iodide spot was developed. Since all samples received identical amounts of radioiodide, the size of the spot on the autoradiogram indicated only qualitative differences in the amount of iodide present. However, in the absence of sufficient iodide to localize in a spot, the carrier-free I^{131} failed to localize and radioactivity could be detected along the entire route of movement.

Fig. 2 is a reproduction of a typical chro-

[¶] In another series I^{131} was added to unprocessed urine before it was dried, to check volatilization of iodide.

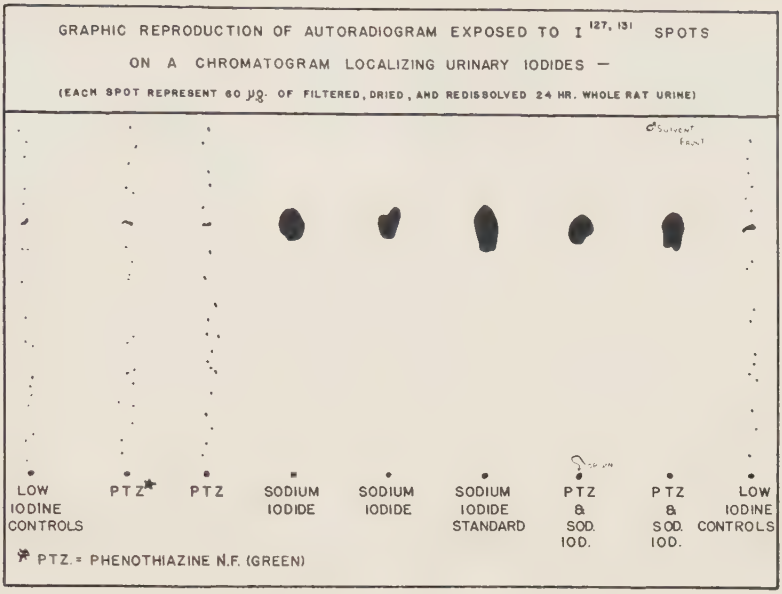
GRAPHIC REPRODUCTION OF AUTORADIOGRAM EXPOSED TO I 127, 131 SPOTS
ON A CHROMATOGRAM LOCALIZING URINARY IODIDES —
(EACH SPOT REPRESENT 60 µg. OF FILTERED, DRIED, AND REDISSOLVED 24 HR. WHOLE RAT URINE)
Solvent Front
Origin
LOW IODINE CONTROLS
PTZ*
PTZ
SODIUM IODIDE
SODIUM IODIDE
SODIUM IODIDE STANDARD
PTZ & SOD. IOD.
PTZ & SOD. IOD.
LOW IODINE CONTROLS
* PTZ. = PHENOTHIAZINE N.F. (GREEN)

FIG. 2.

matogram. Due to the background of colors produced by this method of developing the iodide spots, an actual black-and-white photograph was impractical. These chromatographic studies indicated only traces of iodide in the urine of the control animals maintained on low-iodine test diet, and no increase in the animals given 200 mg of phenothiazine N.F. (green) containing 800 μg of organically bound iodine. Animals given 800 μg of iodide as NaI and those given both a combination of phenothiazine N.F. (green) and the NaI showed marked and similar increases in urinary iodides. This experiment was repeated, using a total of two animals in each category, and substantiates the fact that iodine in phenothiazine is not immediately released and consequently is not available in circulation as a free iodide ion.

Discussion. The studies reported here indicate that the inhibition by phenothiazine N.F. (green) of the thyroid uptake of I^{131} cannot be due entirely to its iodine content. Purified forms of the drug show an inhibiting activity far out of proportion to the amount of iodine present. The failure of large amounts of orally administered phenothiazine (commercial form) to increase the renal excretion of iodide within the first 24 hours suggests that iodine is not readily released from its organic combination. It can only be concluded therefore that the administration of phenothiazine N.F. (green) does not increase the circulating iodide content of the blood sufficiently to produce the immediate suppression of thyroid uptake of I^{131}.

The only other explanation which could attribute the thyroid uptake suppression to inorganic iodide saturation would be to suppose that the iodine was broken from its organic nucleus after entering the thyroid. This necessitates two assumptions, both of which appear improbable: first, in order to account for the uptake of sufficient iodide in this manner it must be assumed that the thyroid preferentially localizes the unknown organic molecule to the same degree as it does inorganic iodide; secondly, it must be assumed that the thyroid is able to utilize organic iodine from this compound in a manner not at this time known to occur with other iodinated compounds.

This study does not preclude the fact that iodide ions are not slowly released in the metabolism of the drug by the body and at a later time produce their effect upon the thyroid, possibly in the prevention of the hypertrophy of the gland. It is also conceivable that iodine is an essential component of an organic molecule able to suppress the uptake of radioiodine. It strongly suggests that there is in phenothiazine N.F. (green), probably existing as an impurity in the drug, an unknown organic compound or group of compounds which are capable of suppressing the uptake of radioiodine by the thyroid. Work is progressing to ascertain the actual nature of the causative agent.

Summary. 1. The effect of free iodide (NaI or KI) and phenothiazine N.F. (green) on the suppression of I^{131} uptake by thyroid in rats is studied and compared. Various related compounds and derivatives of phenothiazine are also surveyed. 2. Column chromatographic fractionation of phenothiazine N.F. (green) reveals that the activity described is strongly associated with its iodine content; however, phenothiazine N.F. (purified) with negligible amounts of iodine has some suppressing quality. 3. In continuous feeding experiments phenothiazine N.F. (green) when given in doses sufficient to partially suppress I^{131} uptake did not affect the weight of the thyroid gland, total body weight or any of the other organs studied. No component of phenothiazine N.F. (green) is goitrogenic. 4. A chromatographic method is described for studying the renal excretory levels of free iodide after rats are treated with NaI, phenothiazine N.F. (green) or a combination of both. Those animals receiving NaI or NaI and phenothiazine N.F. (green) showed an equal but elevated level of iodide excretion. Those rats given phenothiazine N.F. (green) alone had little or no iodide in their urine over a 24-hour period. 5. It is concluded that the effect of phenothiazine N.F. (green) on reducing the uptake of I^{131} by the rat thyroid is not due solely to the availability of free iodide to this gland as a result of dissociation from an organic combination.

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Tocopherol Prophylaxis in Multiple Exposure to Hypoxia.* (21320)

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The tocopherols have long been of interest to investigators concerned with factors influencing growth and general well-being of the individual. Many qualities have been attributed to undifferentiated tocopherols, perhaps one of the more controversial and interesting is the anti-oxidant character of the vitamin. Increased oxygen uptake of muscle from tocopherol deficient rabbits was first shown *in vitro* by Victor(1). These findings on the rabbit were confirmed by Madsen(2), Friedman and Mattill(3), and Hummel and Basinski(4). Intact animal studies on rats relating tocopherol deficiency to increased oxygen uptake have been made by Telford *et al.*(5), Biddulph and Meyer(6), and Kaunitz and Pappenheimer(7). Friedman and Mattill(3) reported that 6-months-old rats reared on a tocopherol deficient diet showed a 40% greater oxygen uptake by skeletal muscle than muscle from rats on a normal diet. They also reported that 13-months-old tocopherol deficient

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rats eliciting considerable dystrophy showed a much smaller oxygen uptake, tending more toward the normal level. Houchin and Mattill (8) reported that oxygen consumption by dystrophic hamster muscle is as much as 250% higher than normal. Hove *et al.*(9) first observed in their studies on adult rats under anoxia conditions that tocopherol deficient animals had less anoxic anoxia resistance than did tocopherol supplemented animals. Later, Puig Muset and Valdecasa(10) concurred in general with these findings.

Encouraged by these interesting data, a series of experiments was designed to elucidate the role of alpha tocopherol in hypoxia.

Method. Young Dutch Belted rabbits of both sexes were selected as the test animals. Litter mates were divided into 3 diet groups, a synthetic diet, deficient in tocopherol,‡ the same diet supplemented with 4 mg of synthetic alpha-tocopherol§ three times weekly; and a normal diet.‖ All were maintained on isocaloric diets with the deficient group furnishing the base line in the paired feedings. All animals received 2 mg ascorbic acid daily. They were kept in quarters conditioned so that a relatively constant temperature (75-80° F.) and humidity were maintained. They were in good health except for the influence of their particular diets. After 28-35 days the rabbits on the deficient diet began to exhibit signs of tocopherol deficiency as shown by an acute weight loss and the typical slowed body righting reflex. When these two conditions developed the animals were placed in individual cages within a decompression chamber (22″ x 34″). For the second group of animals, a period of 28 days of alpha tocopherol supplementation was the minimum before placing them in the decompression chamber. The third group was kept in their quarters on the normal diet for the same length of time as the second group before placing in the decompression chamber. The decompression chamber was evacuated at a constant rate (simulated 2500 feet per minute) to 240 mm Hg (simulated altitude of 30000 feet) and maintained at this pressure during the exposure period. The chamber was ventilated by allowing approximately 10 liters of room air per minute to enter during the decompression period.

TABLE I.
Low Pressure Exposure Data Group "A."

Diet	Rabbit No.	1st exposure	2nd exposure	3rd exposure
		Min.		
Deficient	30	60	15 died	
	43	300	121	209 died
	54	340	105 died	
	32	345	263	156 died
Normal	50	280	387	420
	52	340	375	410
Supplement	31	114	420	420
	60	238	420	420
	44	350	185 died	
	53	375	420 sacrificed	
	28	400	371	182

Ascent was without incident and state of consciousness was routinely determined, at periodic intervals, by giving the animal an electrical stimulus of 1-3 volts, which was adequate to elicit a head movement response. The stimulus was delivered through two electrodes, one placed in each external ear of the rabbit.

Results. The problem was divided into two general groups: (A) the preliminary survey group and, (B) the principal group.

In the preliminary survey group, 11 animals: 4 deficient synthetic, 5 supplement synthetic, and 2 normal diet animals were subjected to 3 decompression experiences (Table I) of varying length.

It should be observed that of the four deficient diet animals, 2 died during the second decompression experience and the remaining 2 died during the third exposure. The two normal diet animals survived 3 decompression

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‡ The synthetic diet had the following composition:

casein	20	brewers yeast	10
cellulose flour	15	cod liver oil	2
sucrose	5	Hubbell's salt mixture	4
corn starch	41	lard	3
			100

§ Gratuitously supplied by Merck and Co.

‖ Purina Rabbit Chow Checkers contain 103 p.p.m. total tocopherol, according to Ralston Purina Co. analysis. Purina Rabbit Checkers used in our feeding showed 140 p.p.m. undifferentiated total tocopherol, according to Distillation Products Industries. (Communication from Dr. Phillip L. Harris.)

TABLE II. Mortality-Survival Data Group "B."

	Deficient synthetic diet	Normal diet	Alpha-tocopherol supplemented synthetic diet
Died during first chamber experience	18	12	7
Died during 2nd chamber experience	2	2	1*
Died during 3rd chamber experience		2	0
Survived 3 chamber experiences	0	5	10
Total	20	21	18

* Died in animal room of unknown causes before second chamber experience.

experiences. Of the 5 supplement animals one died during the second decompression experience, one was sacrificed after the second decompression exposure and the remaining 3 survived the 3 exposures.

In the principal group, 59 animals: 20 tocopherol deficient synthetic, 21 normal, and 18 alpha-tocopherol supplement synthetic diet animals were decompressed 240 mm Hg (30000 ft. simulated altitude) and stimulated at 5 minute intervals for 7 hours and then recompressed to the existing atmosphere pressure in 10 minutes. They were returned to the animal room cages and the survivors of the first decompression experience were subjected to similar 7 hour chamber experiences on the next 2 consecutive days. (Table II).

During the first decompression 18 of the 20 (90%) tocopherol deficient synthetic diet animals died as compared with 12 out of 21 (57.1%) in the normal diet group, and 7 of the 18 (38.8%) in the alpha-tocopherol supplemented group. During the second decompression experience the remaining 2 (10%) of the tocopherol deficient synthetic diet animals and 2 (9.5%) of the normal diet animals died. One animal (5.7%) of the alpha-tocopherol supplemented synthetic diet died in the animal room cage prior to its second decompression experience.

During the third decompression exposure, 2 (9.5%) of the normal diet group died (all had died in the deficient group at this time) and none died in the alpha-tocopherol supplemented synthetic diet group.

Summary and conclusions. 1. With reference to "A" group, all tocopherol deficient animals died during the course of the 3 decompression exposures. None of the normal diet group, and only one of the tocopherol supplemented animals died. 2. Group "B". No deficient diet animal survived 3 consecutive decompression experiences under the conditions of this experiment while 23.9% of the normal diet group survived these 3 experiences and 35.5% of the alpha-tocopherol supplemented synthetic diet group survived. 3. It can therefore be concluded that rabbits deficient in tocopherol to a dystrophic level did not elicit as much resistance to the hypoxic factor as did those animals on the normal or tocopherol supplemented diet. Those animals on the tocopherol supplemented diet had a greater survival rate (11.6%) than did those on the normal diet.

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Wound Healing and Mortality After Total Body Exposure to Ionizing Radiation. (21321)

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The extensive physiological disturbances produced by total body exposure to ionizing radiation might well be expected to retard the healing of a wound received concurrently. Special interest attaches to the combination of traumatic injury and ionizing radiation injury because it would be seen in many patients following an atomic explosion. Radakovich and associates(1) studied the rate of restoration of a surgically produced defect in the skin and superficial fascia of rats; they found no retardation of closure after 150 r or 450 r total body irradiation, and the wounds of animals surviving 650 r closed slightly faster than controls.

The present experiments demonstrate that an LD_{50} of x-ray retards the attainment of tensile strength in healing wounds, but only slightly.

Method. Male Swiss albino mice aged 8½ to 10½ wk were used. The healing-rate experiment was done 3 times. Each time, 60 to 100 animals were housed in a large metal cage from the time of weaning until use, when they were assigned at random to either the irradiation group or the sham-irradiation group. The whole body x-ray dose was 500 r, delivered with a 250 kvp therapy unit by a technic previously described(2). The sham-irradiations were carried out exactly as the actual irradiations, except that the x-ray tube was not energized. Immediately after irradiation or sham-irradiation, the mice were weighed and anesthetized (sodium pentobarbital, 0.072 mg/g I. P.), and their abdomens were shaved. The standard wound was then made, as a longitudinal incision through all layers of the abdominal wall just to the left of the midline in the upper quadrant. The wound was measured immediately after its production and the length recorded; average wound length was 11.8 mm, with a range of 10 to 15 mm. The wound was closed with 2 interrupted sutures of 00000 silk. All wounds were made by one operator and all closures by another operator. At the time of surgery the operators did not know which mice had been irradiated and which sham-irradiated, since anesthetized mice from both groups were supplied to them at random by a third person. The sutured mice were separated into 6 groups by means of a table of random numbers. Each group was then housed in glass jars, with approximately 3 irradiated and 3 non-irradiated mice in each jar. One group was used on each wound strength test day, the test days being post-operative days 2, 4, 6, 8, 11, and 14. The *tensile strength* of the wounds was determined by the method described by Kobak and associates(3). The mouse was sacrificed (ether) and the sutures removed. A thin rubber balloon was introduced into the peritoneal cavity through a suprapubic puncture wound. The balloon was inflated and the intra-abdominal pressure at which the wound disrupted was recorded with a mercury manometer. The pressure was increased at a rate of 3.5 mm of mercury per second, controlled by inflating the balloon at such a rate that the manometer pen rising on the kymograph drum followed a predetermined line. A separate mortality experiment was done, in which 93 mice were randomized into 3 groups; one group received the standard irradiation and wound, one group sham-irradiation and wound, and one group irradiation only. These mice were observed for mortality for 30 days.

Results. The mean tensile strength attained by the wounds on each test day is shown in Table I and plotted in Fig. 1. It is evident that from day 6 to day 11 the healing of the wounds in the irradiated animals is significantly retarded, but not by more than 3 days.

By the 14th day, the wounds had at-

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TABLE I. Mean Wound Strength on Various Post-Operative Days.

	Control			Irradiated			
Day	No. of mice	Mean wound strength (mm-Hg)		No. of mice	Mean wound strength (mm-Hg)		p† for difference
2	16	61.3	3.1*	15	62.4	2.9	.8
4	19	79.7	5.3	16	85.6	5.8	.5
6	16	124.1	9.6	17	98.5	5.5	.03
8	17	182.5	9.4	14	129.6	10.6	.001
11	11	215.3	10.2	10	171.8	18.8	.05
14	15	231.5	8.2	10	220.0	17.9	.5

* Stand. dev. of mean.

† Probability that difference observed between control and irradiated might be due to chance alone.

tained a strength close to that of the rest of the abdominal wall, and in 40% of irradiated and 47% of sham-irradiated animals the abdominal wall separated in some area other than the wound. For this reason observations were not made at longer times. When some portion of the abdominal wall other than the wound separated, the maximum pressure attained was taken as the wound tensile strength. On days 2 through 11 almost all separations occurred as sudden disruptions of the wounds. There were a few small stitch abscesses in both irradiated and sham-irradiated animals, but otherwise there were no gross wound infections.

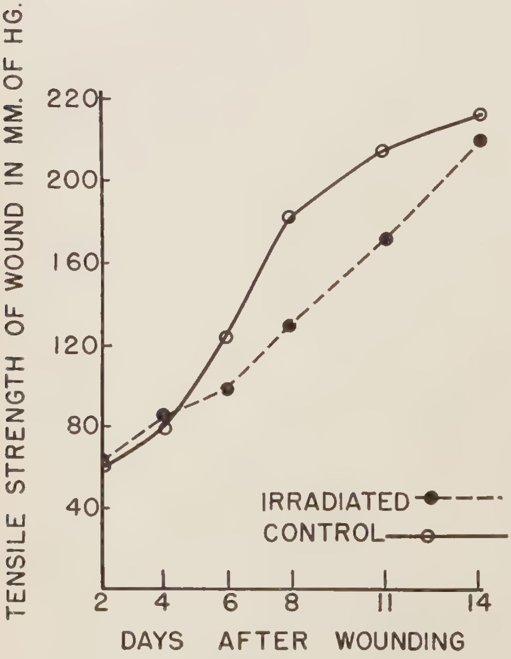

FIG. 1.

Mean wound strength vs. days after wounding.

The healing curves for the 3 repetitions of the experiment showed some variation, but the difference between irradiated and sham-irradiated animals was of about the same magnitude in each repetition. The data shown are the pooled results from all three repetitions. Analyses of the wound strength data for possible relationship to wound length, to initial weight of mouse, or to the number of sutures intact at sacrifice indicated that none of these factors had any correlation with the strength attained by the wound.

The results of the mortality study are shown in Table II.

Discussion. The results indicate that clean wounds closed promptly, in mice, may show some diminution in healing rate when complicated by ionizing radiation injury, but that the total healing time is not increased by any large factor over that of similar wounds without irradiation.

The deleterious action of radiation upon wound healing may be due to the altered physiology that follows total body irradiation, or to local action upon the wounded tissues. Lawrence and associates(4) showed that local irradiation of the wound site just before wounding can produce greater retardation in healing than that shown here; their local dose was 2000 r, however.

The mortality study showed a similar post-irradiation mortality in wounded and in unwounded mice, and it is evident that wound and irradiation show no such synergism as Brooks and associates(5) or Baxter and associates(6) found with burn and irradiation. The synergism these authors found disappeared when antibiotics were administered in the

TABLE II. Results of Mortality Study.

	No. of mice	Deaths, day 0-3	Deaths, day 4-30	30-day mortality, %
500 r without wound	30	0	15	50.0
Wound + sham-irradiation	32	4	1	15.6
Wound + 500 r	31	3	12	48.4

post-irradiation period. Gustafson and Cebul (7) exposed dogs to 200 or 300 r whole body radiation; resection of 10 cm of ileum 20 hr later did not raise the mortality significantly.

Summary. A standardized laparotomy wound was produced in mice following a whole body x-ray dose of 500 r. The tensile strength of the healing wounds was compared on several post-operative days to wounds in unirradiated mice. In addition, 30 day mortality was determined for mice subjected to wounding, to irradiation, and to wounding plus irradiation. Irradiated animals showed a significant retardation of wound healing from the 6th through the 11th day after wounding, but not thereafter. Wounding did not alter post-irradiation mortality.

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Serum Lipoproteins in Experimental Diabetes. III. Effect of Anterior Pituitary Growth Hormone.* (21322)

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Changes in serum lipoprotein occurring in depancreatized dogs during ketosis and following heparin administration have been described previously(1,2). Since the anterior pituitary growth hormone (GH) has a diabetogenic effect(3,4), influences the activity of the islets of Langerhans(5,6), and causes lipid mobilization(7-9), it was thought that GH may influence serum lipoprotein. This possibility was investigated in normal and depancreatized dogs.

Materials and methods. Six mongrel dogs were used in these experiments. Four normal dogs were fasted for about 18 hours. After securing a venous blood sample, purified growth hormone (Armour Lot 5 PWR)‡ was injected intramuscularly (2 or 3.5 mg/kg freshly dissolved in NaOH at about pH 9). Blood samples were again obtained 3 and 6 hours after the injection. The dogs were then fed a single meal of commercial dog food. Another fasting blood sample was obtained 24 hours after the injection. The injections of GH were continued for 5 to 10 days and blood samples taken every second or third day. Blood glucose was determined in duplicate according to Nelson(10). Serum aliquots were studied by means of electrophoresis on filter-paper and cholesterol and lipid phosphorus content of the serum and of the electrophoretic fractions were determined as previously described(1). A control experiment was performed on a normal dog injected with dilute NaOH. One depancreatized diabetic dog was also treated with GH (2 mg/kg) according to

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‡ Gift of Dr. I. Bunding of the Armour Laboratories.

TABLE I.

Dog	Time	Cholesterol, mg/100 ml serum		Lipid phosphorus, mg/100 ml serum		Blood glucose, mg %	Total GH dose, mg
		TOT	A_{α_1}	TOT	A_{α_1}		
A	0	236	195	8.0	6.9	115	
	3 hr	222	184	7.6	6.2	123	31.5
	6 hr	220	184	8.0	7.4	115	
	1 day	252	213	7.8	6.7	115	
	3 days	308	266	10.4	8.7	96	94.5
	5 days	435	384	11.0	9.0	148	157.5
Final change, %		+84%	+95%	+37%	+30%		
B	0	258	209*	10.8	8.9*	80	
	3 hr	242	195*	10.3	7.8*	112	37.0
	6 hr	234	180*	9.7	7.5*	112	
	1 day	234	184*	10.3	8.0*	112	
	2 days	250	206*	10.6	9.3*	112	74.0
	5 days	290	253*	10.0	8.7*	112	148.0
	7 days	381	307*	11.5	9.7*	112	22.0
Final change, %		+47%	+47%	+6%	+9%		
Y	0	228	178	11.3	10.0	75	
	3 hr	223	168	11.2	8.5	75	38.0
	6 hr	201	165	9.3	7.9	83	
	1 day	219	173	10.4	7.9	83	
	3 days	295	251	11.8	9.8	75	114.0
	7 days	332	261			58	228.0
	9 days	430	355	11.8	7.7	103	304.0
Final change, %		+88%	+99%	+44%	–23%		
W	0	300	132	11.2		89	
	3 hr	282	134	11.6		89	25.0
	6 hr	266	114	11.2		89	
	1 day	206	160*			89	
	5 days	247	131			96	125.0
	7 days	223	164			96	150.0
	12 days	207					250.0
Final change, %		–25%	+24%				
AU	0	276	225	14.1	10.8	169	
(diab.)	3 hr	228	173	10.3	7.8	46	20.0
	6 hr	143	98	10.5	7.9	31	
	1 day	229	138	14.7	10.4	246	
	10 days	272	225	10.8	9.5	364	200.0
Final change, %		–1.4%	0	–23%	–12%		

* Includes the A_{α_2} fraction.

the schedule followed for the normal dogs. This dog received a constant diet and a maintenance dose of protamine zinc insulin as described previously(11). The statistical significance of the results was calculated with the method of Fisher(12).

Results. (Table I.) Carbohydrate utilization of normal dogs treated with GH was not markedly impaired. Fasting blood sugar levels remained within normal range, although slightly elevated above the starting values. Traces of sugar appeared, at times, in the urine toward the fifth day of treatment. The intravenous glucose tolerance curve of dog W, after 250 mg of GH in 12 days, was normal. On the other hand, the administration of GH caused a temporary hypoglycemia followed by a marked hyperglycemia in the depancreatized dog. Total serum cholesterol decreased significantly ($P < 0.01$) during the first 6 hours after GH administration in all animals. In 3 out of 4 normal dogs, this was followed by a marked and significant ($P < 0.05$) rise resulting, after 5 to 9 days, in a cholesterol level higher than the initial one.

Total lipid phosphorus behaved like cholesterol, but the changes were not quite as marked. In spite of the elevated lipid content, the turbidity of the serum did not increase. The albumin-alpha$_1$ lipoprotein fraction, which contains most of the lipids of normal dog serum, showed changes in cholesterol content parallel to and sufficient to account for those of the total serum lipids. The concentration of lipid phosphorus in this fraction varied in a similar manner and, although the changes were less marked, they were statistically significant ($P<0.05$). The lipid content of other electrophoretic fractions was similar to that noted in previous experiments of this series(1,2) and did not vary significantly following GH administration. Serum lipids and lipoproteins of the diabetic dog behaved like those of the normal. No changes of serum lipids were noted in the control dog injected with NaOH ruling out the possibility that some of the observed changes may have been non-specific results of the experimental procedure or of fasting.

Discussion. In the normal dog, the administration of GH causes an initial decrease in cholesterol and lipid phosphorus content of serum. This decrease occurs principally in the fast-moving lipoprotein fractions (albumin-alpha$_1$ globulin), subsides after a few hours and is followed by a gradual rise. After a few days, the concentration of cholesterol and lipid P returns to or rises above the initial levels. Greenbaum and McLean(8,9) found that the plasma lipids of normal rats rise shortly after the administration of GH, returning later to the initial levels. The authors believe that GH causes a mobilization of fat from the depots to the liver, followed by an increased fat catabolism. This may be true in the dog also with the difference that, in this species, the increase in lipid uptake by the liver or the peripheral tissues may precede the mobilization of depot fat, and thus the rise in serum lipids would be preceded by a fall. In the normal dog, after GH administration, as well as in the depancreatized dog under insulin control(1), practically all the changes in lipid concentration occur in the albumin-alpha$_1$ fraction. Since this fraction is believed to be the end result of the metabolic conversion of serum lipoproteins(13), these results suggest that GH stimulates lipid transport and metabolism. The solubility of the albumin-alpha$_1$ fraction is relatively high, which may explain the failure of the serum to become more turbid in spite of the increased lipemia. In contrast, in the decompensated diabetic dog, the sharp rise in the lipid content of the slower migrating fractions, accompanied by increased turbidity, indicates that a metabolic block probably exists(1). The absence of any significant impairment of carbohydrate utilization in the normal dogs treated with GH suggests that the described effects of GH are not due to its diabetogenic action. In agreement with this hypothesis, the changes in serum lipids caused by GH were less marked in the diabetic animals than in the normal dogs, in spite of the aggravation of the diabetic state.

Summary. Administration of pituitary growth hormone to 4 normal and to 1 depancreatized dog produced a decrease in the lipid content of the serum and of the albumin-alpha$_1$ lipoprotein fraction within the first 6 hours. After repeated daily injections of GH for 5 to 10 days, a progressive rise in the lipid content of this fraction took place and resulted, in some animals, in a marked hypercholesterolemia. No evidence of impairment of carbohydrate utilization was found in normal dogs, while fasting blood sugar and glycosuria increased in the diabetic animal. The possible significance of these findings is discussed.

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Effect of Methyl-Bis (Beta-Chloroethyl) Amine on Aerobic Glycolysis of Thymus Lymphocytes.* (21323)

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Schrek has shown that nitrogen mustards, as well as a commercial adrenocortical extract, Lipo-Adrenal Cortex (L.A.C.), are cytolytic to thymus lymphocytes *in vitro*(1). Our efforts to correlate the lymphocytolytic action of L.A.C. with a characteristic change in the metabolic activity of the cells affected have led to the observation that L.A.C. stimulates the aerobic glycolysis of thymus lymphocytes. This stimulation is relatively specific, and occurs at concentrations which have no significant effect on respiration or anaerobic glycolysis(2-4). We have therefore investigated the influence of one of the nitrogen mustards, methylbis (β-chloroethyl) amine (MBA)† on the aerobic glycolysis of thymus lymphocytes to determine whether its effect on aerobic glycolysis is similar to that of L.A.C.

Experimental. Rat thymus lymphocytes were suspended in Ringer-Krebs phosphate buffer as previously described(3,4). One ml of cell suspension was incubated at 37°C in O_2 with 0.02 M glucose and freshly prepared MBA,‡ in a final volume of 1.5 ml. Incubations were carried out in a Dubnoff Metabolic Shaking Incubator(5), operated at low speed to minimize mechanical damage to the cells. Protein was then precipitated with 10% TCA, and lactic acid was determined in the supernatant by the Miller-Muntz method(6,7). The results are expressed as % stimulation of aerobic glycolysis.

Results. Table I shows the results obtained in 3 typical experiments with MBA at concentrations ranging from 10^{-5} to 10^{-3} M. Stimulation of aerobic glycolysis was observed at 10^{-4} and 10^{-5} M, and inhibition at 10^{-3} M. The degree of stimulation produced by L.A.C.§ in these experiments is also indicated. We have not found a concentration of MBA which stimulates to the same degree as L.A.C.

TABLE I. Effect of Methylbis (β-chloroethyl) Amine and L.A.C. on Aerobic Glycolysis of Thymus Lymphocytes.

Experiment	$Q_A^{O_2}$*	% stimulation or† inhibition
Control	1.8	—
10^{-5} M MBA	2.4	+33
10^{-4} "	2.2	+20
10^{-3} "	1.4	−25
L.A.C.‡	3.5	+93

* Aerobic glycolysis.

† Plus signs represent stimulation, minus signs, inhibition.

‡ .05 ml Lipo-Adrenal Cortex in cottonseed oil added to 1.5 ml final vol.

Avg of three separate experiments.

Tri-ethylenephosphoramide (TEPA),||¶ which, like MBA, possesses the ethylenimine structure, had no effect on aerobic glycolysis at concentrations from 10^{-3} M to 10^{-6} M. Furthermore, bis-β-hydroxy-ethyl sulfoxide and 2, 2′ thio-diethanol, which have a structure somewhat similar to MBA, had either no effect or inhibited slightly at this concentration range.

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* This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

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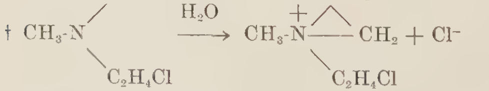
$CH_3\text{-}N(C_2H_4Cl)_2 \xrightarrow{H_2O} CH_3\text{-}\overset{+}{N}(CH_2CH_2)(C_2H_4Cl) + Cl^-$

‡ Obtained through the courtesy of Merck and Co., Rahway, N. J.

§ Obtained through the courtesy of The Upjohn Co., Kalamazoo, Mich.

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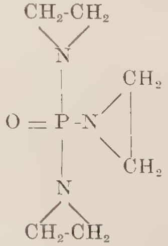

¶ Obtained through the courtesy of Dr. J. H. Williams of the Lederle Laboratories, Pearl River, N. Y.

Discussion. It has been found that the aerobic glycolysis of thymus lymphocytes is greatly increased after exposure to small amounts of L.A.C.(2-4). This increase occurs very rapidly and has been demonstrated within 15 minutes after the cells and L.A.C. are mixed. Longer periods of incubation or larger amounts of L.A.C. are required for lymphocytolysis(8-10). It has therefore been suggested(3), that, if these two phenomena are related, the primary alteration in the cell may be increased glycolysis, and that this is followed by increased permeability to safranin, the index of cytolytic activity. It is of interest that MBA, which also possesses lymphocytolytic activity, similarly stimulates the aerobic glycolysis of thymus cells.

The active grouping of MBA is generally believed to be the quaternary nitrogen ethylenimine structure which forms during hydrolysis(11). TEPA is characterized by three ethylenimine groupings in which the nitrogen is trivalent. These two compounds differ considerably in the type of malignant cell they inhibit. Furthermore, it has been shown that several enzyme systems which are sensitive to MBA are completely resistant to TEPA, even when it is used at considerably higher concentrations(12). The present experiments provide additional evidence that these two types of ethylenimine compounds differ considerably in their selectivity for enzymes.

Summary. At concentrations of 10^{-4} and 10^{-5} M, methylbis (β-chloroethyl) amine stimulates the aerobic glycolysis of thymus lymphocytes *in vitro*. This phenomenon is discussed in relation to the lymphocytolytic activity of this compound.

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Gastrointestinal Absorption and Urinary Excretion of Vitamin B_{12}-Co^{60}.*

(21324)

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The quantitative absorption of orally administered vit. B_{12}-Co^{60} has been utilized as an assay of intrinsic factor present in the gastrointestinal tract. Heinle *et al.*(1) have reported that normal humans excrete less than 25% of an oral dose of 0.5 μg of "vit. B_{12}-Co^{60}" in the feces, while pernicious anemia patients excrete over 75% of this dose. Patients who have undergone total gastrectomy, like individuals with pernicious anemia, also excrete more than 75% of the Co^{60} in the stool following ingestion of labelled vit. B_{12}. The fecal excretion of radioactive material in both these groups reverts to normal if a source of intrinsic factor is administered orally with the vit.(2,3). To avoid the necessity of stool collections, Schilling(4) has proposed a modification of the above procedure which is based on the fact that 30 to 100% of a subcutaneous dose of 1000 μg of vit. B_{12} is excreted in the urine within 24 hours. He administers vit. B_{12}-Co^{60} orally first, and then 2 hours later a "flushing" dose of 1000 μg of unlabelled vit. B_{12}. Theoretically those individuals with in-

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trinsic factor will absorb the radioactive vitamin from the gastrointestinal tract and it will become demonstrable in the urine after the subcutaneous flushing dose is given, while individuals without intrinsic factor will not absorb the radioactive material and therefore not excrete it in the urine.

The above procedures show great promise in the diagnosis of *achylia gastrica* and the differential diagnosis of *megaloblastic anemias* but they have been criticized because of lack of proof that the radioactivity measured in the excreta actually represents vit. B_{12}. There is a possibility of cobalamin decomposition prior to absorption from the bowel. Barbee and Johnson(5) demonstrated in rats that 60% of the orally administered vit. B_{12}-Co^{60} was excreted in the feces in a decomposed form. If such decomposition occurs before absorption, the reliability of procedures depending on a constant bond between vit. B_{12} and Co^{60} would be greatly jeopardized. Our study was initiated to identify if possible the chemical nature of the radioactive material present in the urine, ileal content and stools of patients who had ingested vit. B_{12}-Co^{60}.

Methods. 1. *Urine.* Normal individuals were asked to drink an aqueous solution containing 0.7 μg of vit. B_{12}-Co^{60} with a specific activity of 760 microcuries per mg,[†] followed after 2 hours by a subcutaneous dose of 1000 μg of unlabelled vit. B_{12}.[‡] The container used to administer the radioactive material was counted before and after the dose was given. Urine was collected on ice for 24 hours following ingestion of vit. B_{12}-Co^{60}. Pilot experiments revealed no significant urinary excretion of radioactive material after the first 24-hour period. The radioactivity in a 5 ml aliquot of the urine was measured in quintuplicate and the total radioactivity of the 24-hour output calculated from the average.[§] Measurements of different aliquots from the same urine did not vary by more than ± 5%. This was the same percentage variation as the background. To a 200 ml aliquot of urine was added 170 g of ammonium sulfate and the solution extracted once with 200 ml of n-butanol. In control experiments it was found that this procedure would remove 80% to 90% of the radioactivity if 0.1 μg of vit. B_{12}-Co^{60} was added to urine. The butanol extract was concentrated *in vacuo* to a volume of 3 to 5 ml and the concentrate chromatographed on paper. Previous investigators have used an aqueous butanol system for paper chromatography of vit. B_{12} (6); however, the Rf value in this system is only of the order of .05 which makes separation of vit. B_{12} from other constituents of the urine extracts precarious. In 7:5 mixtures of 2,4-lutidine and water under an ammonia atmosphere (provided by placing dishes containing 10% aqueous ammonium hydroxide in the chromatographic chamber) vit. B_{12} was found to have an Rf value of .45 to .55 and this system was well suited for our purposes. Whatman No. 1 filter paper was spotted with 25 ml of urine concentrate and 50 ml each of vit. B_{12} and vit. B_{12}-Co^{60}, and developed for 15 to 16 hours in the water-lutidine system. The position of the vit. B_{12} control spots each containing about 50 μg was easily discernible by their red color. To determine the location of radioactive material the papers were scanned with a monitor. To quantitate the radioactivity in a given area the strips corresponding to the unknown urine and vit. B_{12}-Co^{60} were cut from the paper and measured in the well scintillation counter. The materials to be chromatographed were applied to the paper in butanol solution. In all patients on whom the urinary excretion was studied, stools were collected also until fecal passage of radioactive material ceased. 2. *Ileal Content.* This investigation was carried out on a surgical patient who was provided with a Grafton Smith long intestinal tube(7) reaching to the low ileum. The tube was placed at the time of laparotomy. After oral intake in this patient had been well re-established but while his intestinal tube was still *in situ,* (as proven by x-ray), the patient was given 0.7 μg of vit. B_{12}-Co^{60} and 1000 μg of unlabelled vit. B_{12} in 30 ml of water by mouth. Ileal contents were collected by continuous Wangensteen suction for

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[†] Kindly supplied by Dr. Charles Rosenblum of Merck and Co., Rahway, N. J.

[‡] Cyanocobalamin in isotonic sodium chloride with Benzyl Alcohol 1.5%.

[§] All samples were counted in 5 ml patent lip vials in a well scintillation counter under constant geometry.

TABLE I. Fate of Radioactivity following Oral Administration of B_{12}-Co^{60}, 3 Patients.

Ingested dose of B_{12}-Co^{60} in C.P.M.	Total excretion of radioactivity in urine in C.P.M.	Total excretion of radioactivity in stool in C.P.M.	% of absorbed dose recovered in urine
———	— × 1000 —	———	
498	145	150	38.8
495	125.2	125.9	33.9
497	101.4	200.5	34.1

48 hours following the oral test dose. 1500 ml of a dark green fluid was collected. To a 100 ml aliquot of ileal content was added 75 g of ammonium sulfate and the mixture extracted once with 100 ml of n-butanol. 3. *Stool.* This experiment was carried out on a normal human. An oral dose of 0.7 μg of vit. B_{12}-Co^{60} and 1000 μg of vit. B_{12} were administered and stools collected for 7 days after which time the feces became devoid of any detectable radioactivity. Most of the radioactive material was excreted in the first 3 days. Aliquots of the homogenized stools excreted from 0-24 hours, 24-48 hours and 48-168 hours were slurried with 100 ml of water containing 75 g of ammonium sulfate. The mixture was then extracted once with 100 ml of butanol.

Results. The 3 normal individuals excreted between 20% and 29% of the orally administered dose of Co^{60} in the urine within the first 24 hours, corresponding to 34% to 39% of the absorbed dose. We wish to emphasize that following ingestion of vit. B_{12}-Co^{60} detectable quantities of radioactive material will appear in the urine only after a flushing dose of the unlabelled vitamin has been given subcutaneously. The percentage of radioactive material absorbed was calculated by subtracting the fecal excretion from the oral dose (Table I). The quantity of radioactive material isolated from the urine is within the range reported by other workers when 1000 μg of vit. B_{12} is given subcutaneously(8).

A single extraction of the urine with an equal amount of n-butanol removed between 78% and 92% of the radioactive material. The efficiency of the extraction process of radioactive material from urine was of the same order as that from extraction of labelled vit. B_{12} from water (Table II). If the urine was incubated at room temperature for 2 weeks the butanol extractable fraction dropped to 51% (urine of E.F.) indicating the possibility that bacterial action had changed some of the vit. B_{12} into non-extractable substances (Table II).

Chromatography of the concentrated urine extract demonstrated that more than 95% of the radioactive material which it contained had an Rf value which was indistinguishable from that of vit. B_{12}. Chromatography of a mixture of vit. B_{12}-Co^{60} and unlabelled vitamin showed that over 90% of the radioactivity was within the area of the red colored spot. Approximately 10% of the radioactivity was detected, diffusely spread, between Rf .00 and .50. Similarly chromatograms of urine extracts showed a diffuse spread of radioactivity proximal to the location of vit. B_{12} amounting to 5% or less of the applied material. These data suggest strongly that the radioactivity in the urine is present as vit. B_{12}-Co^{60}.

The 24-hour sample of ileal contents contained 82% of the ingested Co^{60}. However, n-

TABLE II. Results of Extraction of Urine, Ileal Content and Stool for Vit. B_{12}.

Sample	Patient	Total activity in sample in C.P.M.*	Total activity in butanol extract in C.P.M.	% of activity in butanol extract
1. Known aqueous sol. of B_{12}-Co^{60}	—	84055	75000	89
2. Urine†	E.F.	16000	13944	87
3. ”	J.F.	21452	16720	78
4. ”	C.S.	18440	17120	92
5. ” incubated room temp. 2 wk	E.F.	16000	8160	51
6. Ileal content‡	G.C.	29920	11765	39
7. Stool	E.W.	16399	1307	8

* Vit. B_{12}-Co^{60} had activity of approximately 700000 C.P.M./μg as measured on our counter.

† Urine from patients who received oral B_{12}-Co^{60} and parenteral vit. B_{12}—1000 μg 2 hr following.

‡ Obtained from long intestinal tube in distal ileum.

butanol took up only 39% of the radioactive material in a single extraction (Table II). Eighty-eight % of the ingested vit. B_{12}-Co^{60} was recovered in the 6 day stool collection, but only 8% was extracted into butanol (Table II).

The difference in the extractability of the radioactive material from fresh urine on the one hand and aged urine, ileal content and stool on the other may indicate that vit. B_{12} is changed by bacterial action.

The results of these studies are consistent with the view that urinary excretion of Co^{60} can serve as a measure of absorption of vit. B_{12}-Co^{60} from the intestine.

Summary and Conclusions. 1. Following oral administration of vit. B_{12}-Co^{60} to normal individuals and subsequent "flushing" by subcutaneous injection of cobalamin, the radioactive material could be extracted from urine by n-butanol; the Co^{60} containing substance had the same distribution coefficient between ammonium sulfate saturated urine and n-butanol as authentic vit. B_{12}-Co^{60}. 2. In an aqueous 2, 4-lutidine system the radioactive material in the urine has the same Rf value as pure vit. B_{12} and vit. B_{12}-Co^{60}. 3. Our studies support the view that the Schilling test measures absorption of vit. B_{12}-Co^{60} from the gut rather than absorption of decomposition products. Its use therefore as an assay of intrinsic factor appears justified.

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Propagation of B-Virus in Tissue Cultures.* (21325)

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The isolation of an agent from a human case of ascending myelitis that followed the bite of an apparently "normal" rhesus monkey was reported by Sabin and Wright(1), and was referred to as B-virus. Another strain of the same agent was isolated by Sabin(2), from another fatal human case that occurred under similar circumstances. Although these viruses showed some antigenic relation to the virus of herpes simplex they had several distinctive properties(3a-d). Burnet, Lush and Jackson (4) demonstrated the propagation of B-virus on the chorioallantoic membrane of the developing chick embryo and brought forth further evidence for the antigenic relation between B-virus and herpes simplex virus. More recently Black and Melnick(5) isolated, from the central nervous system of a rhesus monkey, a strain of B-virus which produced a cytopathogenic effect on monkey renal epithelium grown in tissue cultures.

The present report is concerned with studies on the propagation in monkey kidney and in chick embryo tissue cultures of a strain of B-virus isolated from tissue culture fluid of monkey kidney culture. Dr. W. Wood of the Connaught Laboratory in Toronto isolated in his laboratory from the same tissue culture fluid, a similar agent also identified as B-virus. Observations were made on the stability of this virus and on the distribution of the antibody in "normal" rhesus and cynomolgus monkeys.

Materials and methods. The B-virus strain

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* Aided by a grant from The National Foundation for Infantile Paralysis.

TABLE I. Propagation of B-Virus in Roller Tube Cultures of Cynomolgus or Rhesus Monkey Kidney Tissue. Entries indicate proportion of tissue cultures showing cytopathogenic effect.

Calculated dil. of inoculum from source	Tissue culture generation	Cytopathogenic effect in tissue cultures on 4th day after inoculation — Neg. log of virus dil. inoculated								Infectivity of tissue culture fluid tested in rabbits
		1	2	3	4	5	6	7	8	
10^{-0} (1% mouse cord susp.)	1	—	3/3	3/3	3/3*	3/3	1/3	0/3	0/3	‡
10^{-5}	2	3/3	3/3	3/3*	3/3	3/3	3/3	0/3	0/3	
10^{-8}	3	3/3	3/3	3/3	3/3*	3/3	1/3	0/3	0/3	
10^{-11}	4	3/3	3/3	3/3*	3/3	3/3	0/3	0/3	0/3	
10^{-14}	5	3/3	N.T.	3/3	3/3*	3/3	0/3	0/3	0/3	
10^{-18}	6	3/3	3/3	3/3	3/3*	1/3	0/3	0/3	0/3	
10^{-22}	7	3/3	3/3	3/3	3/3	2/3	0/3	0/3	0/3	‡
10^{-24}	8†			3/3	3/3	3/3	3/3	3/3	0/3	
10^{-26}	9†			6/6	6/6	6/6	6/6	5/6	0/6	‡

* 3 cultures pooled and used as inoculum for next passage.
† Inoculated into stationary incubated bottles and harvested on 3rd day.
‡ Undiluted fluid produced paralysis and death following intradermal injection.
N.T. = Not tested.

studied in these experiments was isolated from a pool of fluid from cultures containing fragments of minced kidney tissue from several rhesus monkeys. The cultures had been seeded with a strain (Mahoney) of poliomyelitis virus Type 1. When this fluid was harvested and titrated in roller tubes containing monkey renal epithelium a cytopathogenic effect was observed which appeared to be different from the degeneration caused by poliomyelitis virus(6). Samples of tissue culture fluid from 2 pools[†] were stored in our laboratory at 4°C for approximately 3 months before they were used for inoculation into tissue culture, rabbits and mice. *Tissue cultures* were prepared from kidneys of cynomolgus or rhesus monkeys or 8 to 10 days old chick embryos. The tissue was trypsinized as suggested by Dulbecco(7) and Dulbecco and Vogt(8). Morgan, Morton and Parker's synthetic mixture 199(9) with 1% horse serum served as nutrient fluid. 100 units penicillin and 0.1 mg streptomycin per ml were added. 2 ml of tissue suspension (1 ml packed trypsinized kidney cells or chick embryo cells suspended in 1000 ml medium) were inoculated into roller tubes and incubated in a stationary position at approximately 36° C. Kidney tissue cultures were used after 6 to 8 days and chick tissue cultures after 2 to 3 days. At that time the fluid was changed and the virus dilution inoculated. Following an incubation period of 4 days for cultures of both types of tissue the cytopathogenic effect was determined microscopically and the results recorded.

Results. Isolation of the virus. Fluid from the original pool 56-1 inoculated into tissue cultures produced cytopathogenic changes typical for poliomyelitis virus activity; inoculation into cultures of chick embryo tissue resulted in cytopathogenic degeneration; after intracerebral inoculation of undiluted tissue culture fluid into 4 weeks old CFW mice there appeared tremor, ataxia and prostration, and about 50% died within 5 to 16 days. Two mice with such symptoms were sacrificed on the 6th and 9th days. Their cords were made up separately as a 1% suspension in saline and inoculated into kidney cultures. The tissue changes which developed within 48 hours in the tubes containing the lowest dilution were quite distinct and were neutralized by an anti B-virus serum kindly supplied by Dr. Albert Sabin. Rabbits inoculated intradermally with the original pool as well as with tissue culture fluid from later passages developed local inflammation and necrosis, flaccid paralysis beginning in one hind-leg and extending to all 4 extremities and died 7 to 15 days after inoculation. The pathogenicity of this agent for

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[†] We are indebted to Dr. R. D. Defries and Dr. W. Wood of the Connaught Laboratory in Toronto for supplying us with samples from pool 56-1 and 61-2.

TABLE II. Rate of B-Virus Multiplication in Chick Embryo Tissue Cultures.

Hr after seeding†	Approx. % of tissue degeneration in flask cultures	Infectivity data of fluid in flask culture Neg. log of virus dil. inoc. into roller tubes of chick embryo tissue						
		2	3	4	5	6	7	Titer
0	—	—	8/8*	1/8	0/8	—	—	$10^{-3.6}$
2	—	8/8	4/8	0/8	—	—	—	$10^{-3.0}$
4	—	8/8	4/8	0/8	—	—	—	$10^{-3.0}$
8	—	8/8	2/8	1/8	—	—	—	$10^{-2.0}$
12	—	8/8	4/8	0/8	—	—	—	$10^{-3.0}$
24	—	8/8	2/8	0/8	—	—	—	$10^{-2.7}$
48	20	—	8/8	6/8	2/8	0/8	—	$10^{-4.5}$
72	60	—	—	8/8	6/8	1/8	0/8	$10^{-5.4}$
96	100	—	—	—	8/8	2/8	0/8	$10^{-5.7}$

* Proportion responding.

† B-virus 829/6 5th chick tissue culture passage.

rabbits and mice, the neutralization of cytopathogenic changes in kidney and chick embryo tissue cultures by an anti B-virus serum supported the classification of the isolated agent as a strain of B-virus.

Propagation of B-virus in tissue cultures. Passages of B-virus in kidney cultures were initiated with the cord suspension of one mouse sacrificed 6 days after intracerebral inoculation with fluid from pool 56-1. Three roller tubes were used for each 10-fold dilution and incubated for 4 days, at which time all cultures containing the inoculum of 10^{-3} and 10^{-4} showed complete degeneration. The fluid of one dilution level was pooled and used as an inoculum for the next passage. Evidence for the propagation of the B-virus strain employed in these studies is presented in Table I. Multiplication is indicated by the presence of virus activity in successive subcultures and at dilutions well beyond the range of infectivity titer of the original inoculum. Various levels of tissue culture passages were inoculated into rabbits and mice. Three to 4 months old rabbits received 0.2 ml intracutaneously. Four to 6 weeks old albino mice were inoculated with 0.03 ml intracerebrally or intracutaneously, 0.5 ml was used for intravenous or intraperitoneal inoculation. The animals were observed over a period of 4 weeks. Tissue culture fluid taken from the 9th passage contained approximately 10^8 ID_{50}/ml for rabbits injected intradermally, $10^{7.0}$ ID_{50}/ml in monkey kidney cultures and $10^{7.5}$ ID_{50}/ml for chick embryo tissue. On the other hand, mice responded only irregularly after intracerebral or intravenous inoculation with tremor, ataxia or death; and no signs were observed in mice following intraperitoneal or intradermal injection.

Multiplication of B-virus in chick embryo tissue was determined by titration of samples taken from a flask culture at various intervals. Ten-fold dilutions of these samples were inoculated into roller tubes containing chick embryo tissue cultures.

The results of tests for virus activity in samples drawn at intervals from flask culture of chick embryo tissue are given in Table II. Complete degeneration in the flask culture is observed after 96 hours, and the titer of the fluid harvested at that time was approximately 100-fold greater than at the time of inoculation.

The possibility of propagating B-virus in chick embryo tissue cultures proved helpful in separating this virus from poliomyelitis virus since there is no evidence that the latter produces cytopathogenic changes or multiplies in cultures of chick embryo tissue.

Stability of B-virus in tissue culture fluid. Studies on stability of infectivity at 40° C, –20° C and approximately –72° C were carried out with one tissue culture pool of B-virus. The fluid which had a pH of 7.2 was distributed into 5 ml vials which were tightly sealed with rubber stoppers. The samples were removed from 40° C at daily intervals and stored at 4° C until titrated in kidney tissue cultures. Samples kept at –20° C and –72° C were titrated weekly immediately after thawing. The pool kept at 4° C was frequently titrated over a period of 8 weeks. The rate of

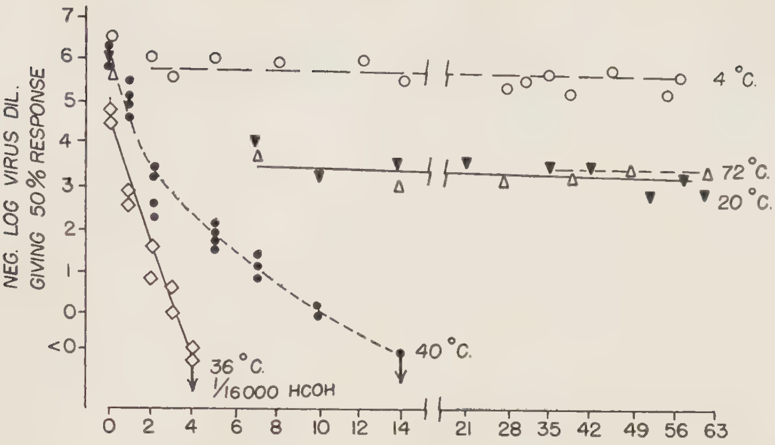

FIG. 1. Stability of B-virus in T.C. fluid at pH 7.2 and at various temp.

inactivation in the presence of formalin was studied at 36° C. The samples were removed from the incubator and stored at 4° C until titrated 7 days after starting the experiment.

The data presented in Fig. 1 show that B-virus stored at 4° C is relatively stable. No significant loss was demonstrable over a period of 8 weeks. Samples frozen at –20° C and –72° C lost approximately 2 logs in infectivity titer after they were frozen once. Repeatedly freezing and thawing up to 3 times thereafter did not measurably reduce the infectivity titer, nor was infectivity measurably affected by storage at these temperatures for a period of 2 months. Storage at 40° C destroyed all demonstrable infectivity within 2 weeks. The rate of inactivation at 36° C at a formalin concentration of 1/16000 was tested in order to obtain some comparison with the stability of poliomyelitis virus inactivated under similar conditions. The zero day sample which was stored at 4° C for one week before titration lost approximately 2 logs compared with the original fluid. This would indicate that there was an inactivating effect of formalin at 4° C. No infectious activity was demonstrable in samples kept for 4 or more days at 36° C. Poliomyelitis virus of similar infectious titer would require approximately 4 times this formalin concentration (*i.e.* 1/4000) in order to be destroyed within 3-4 days.

Antibodies in monkey sera. Neutralization tests in kidney cultures were performed to determine the distribution of B-virus antibody in sera of cynomolgus and rhesus monkeys. All sera tested were diluted ¼ and incubated, at room temperature for 30 minutes, together with equal volumes of B-virus containing 32 to 100 ID_{50} for tissue cultures; 0.5 ml of this mixture was inoculated into each of three roller tubes. If no degeneration occurred in 2 of the 3 cultures, or in all 3, the serum was considered to possess antibody. The range of titer variation in this system was 1:4 - 1:256.

The frequency of occurrence of monkey sera showing neutralizing activity against B-virus is illustrated in Table III. The percentage of positive sera varies between 25% and 30% for the years between 1950-1954. It seems that a relatively high percentage of rhesus monkeys had been in contact with B-virus or an agent antigenically related to it, and that this had occurred at a rather constant rate in the age group and source from which these monkeys were delivered. Antibodies were found in 13 sera from 46 cynomolgus monkeys bled in this laboratory in 1953; however, of 36 cynomolgus monkeys bled in 1954 none showed neutralizing activity. Whether or not cynomolgus monkeys are infected with this or an antigenical similar virus in nature cannot

TABLE III. B-Virus Neutralizing Activity in Rhesus Monkey Sera. Entries are proportions of sera showing neutralizing activity.

Bleeding date		1950	1951	1952	1953	1954
Rhesus monkeys	#	6/20	91/341	23/94	6/23	9/35
	%	30%	27%	25%	26%	26%

be concluded from these observations, since the cynomolgus monkey, may have acquired infection from the rhesus when both were housed in the same enclosure from time to time. Some efforts will be made to clarify this point further.

Discussion. The 2 B-virus strains described by Sabin and Wright and by Sabin had been isolated from fatal human cases following contact with rhesus monkeys. The demonstration of neutralizing antibodies in monkeys strongly suggested that this species may carry the virus without showing signs of disease comparable to those seen in the human cases. Recently this virus has been isolated directly from the CNS of monkeys or from "normal" monkey kidney tissue. If present in tissue culture fluid, intended for the preparation of vaccines, this virus could be expected to be highly infectious for human beings. The stability studies indicate, however, that it is relatively unstable compared with poliomyelitis virus. Infectivity cannot be demonstrated if B-virus in tissue culture fluid is treated under the same conditions employed for the inactivation of poliomyelitis virus by formalin and heat. The high susceptibility of chick embryo cultures for the described agent may prove helpful in addition to, or in place of, the rabbit in assuring the absence of such contamination from tissue culture pools before they are converted into non-infectious antigens.

Summary. 1. A B-virus strain isolated from "normal" monkey kidney was propagated in cultures of monkey kidney and chick embryo tissues. 2. The B-virus strain studied was relatively stable in tissue culture medium when stored at 4° C. Freezing seemed to destroy some activity, but there was no further loss demonstrable on storage at –20°C and –72°C. 3. Approximately 27% of 513 rhesus monkey sera showed neutralizing activity for the isolated B-virus strain.

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Influence of Altered States of Carbohydrate Metabolism on Distribution and Degradation of Insulin-I^{131}.* (21326)

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Previous studies have indicated that information about insulin metabolism may be obtained from studies using the labeled hormone (1). The present report describes measurements of distribution and degradation of insulin-I^{131} in rats under conditions of (a) glucose loading, (b) fructose loading, (c) alloxan diabetes, (d) phloridzinization, and (e) starvation.

Methods. Materials, methods, and procedures have been described in detail(1). In essence, the technic used measured the concentration of the label in tissues, 15 minutes after insulin-I^{131}‡ was given intravenously to rats.

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† U. S. Public Health Service Research Fellow of the National Heart Institute.

‡ The insulin was kindly supplied by Eli Lilly and Co. and iodinated in the Abbott Laboratories at Oak Ridge.

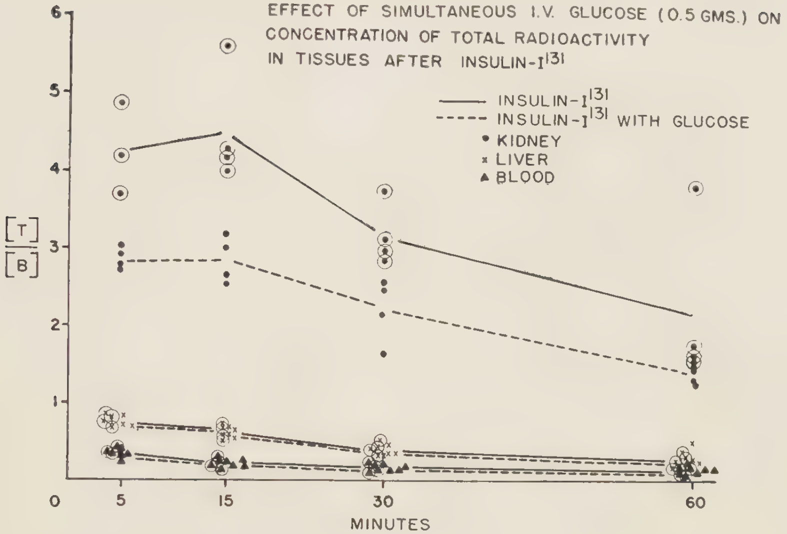

FIG. 1. Values for controls which received insulin-I^{131} only, are circled. Other values are for rats which were given a glucose load intravenously immediately before the insulin-I^{131} was given.

Total tissue radioactivity concentration relative to body wt, $\frac{[T]}{[B]}$, is plotted against time.

Blood, liver, kidney, and muscle were deemed the important tissues to study. The measurement of the degradation of insulin-I^{131} was based on the following observations. Essentially all of the radioactivity of insulin-I^{131} was precipitable by trichloracetic acid (TCA). When insulin-I^{131} was injected into a rat, however, a fraction of radioactivity that no longer had this property rapidly accumulated in tissues and fluids. This was taken to mean that the insulin-I^{131} was degraded, and the loss of TCA precipitable radioactivity over a given time was assumed to be a measure of insulin-I^{131} degradation. The time interval used was relatively short, 15 minutes, to allow time for distribution, yet minimize the overlay of redistribution of labeled degradation products. Male Sprague-Dawley rats kept constantly at 80°F, and fed a standard, vitamin supplemented diet, were used. For each experiment, rats of the same age and weight range were used simultaneously in a control group. Pre-experiment dietary preparation with 10% dextrose in saline was exercised as described previously(1), and all rats received no food or water for 2 hours before use. From 0.47 to 10 μc, (0.005 to 0.02 mg) of insulin-I^{131} in 0.5 ml of 0.9% saline was injected in a tail vein. Fifteen minutes later the rat was exsanguinated and liver, kidney, and gastrocnemius muscle removed. Aliquots of these tissues were minced and homogenized in 10% TCA, and the blood was added to an equal volume of 20% TCA. After centrifugation and one wash with 10% TCA, the 2 fractions, TCA precipitable radioactivity, and supernatant radioactivity, were counted in a well-type gamma counter. The concentration of radioactivity (per cent of the dose per gram of tissue) was determined and multiplied by the dilution factor, $\frac{\text{body wt}}{100}$, giving the ratio of concentration in a given tissue to that initially in the body as a whole. Values of this ratio, $\frac{[T]}{[B]}$, greater than 1 then represented concentrations in that tissue greater than that in the body, and values less than 1 represented concentrations less than that in the body as a whole.

Results. Glucose, 1 ml of a 50% aqueous solution (0.5 g) was given intravenously over a period of 1 minute immediately before the insulin-I^{131} was given. Rats, including controls, were sacrificed at 5, 30, and 60 minutes,

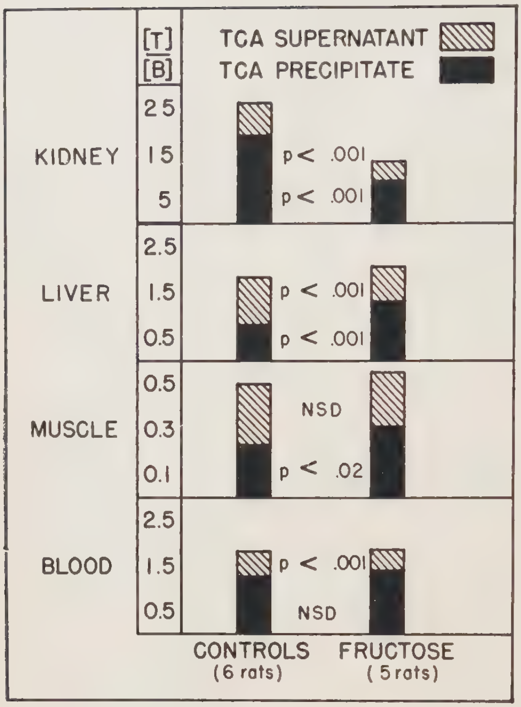

FIG. 2. Comparison of concentrations, $\frac{[T]}{[B]}$, of radioactivity in tissue fractions 15 min. after intravenous insulin-I^{131} was given to control rats and to rats which, immediately before the insulin-I^{131}, were given intravenous fructose. Figures between columns are p values of differences between the fractions in the two adjacent groups. NSD indicates no significant difference.

in addition to the 15-minute interval. The results are shown in Fig. 1. The total radioactivity in liver, kidney, and blood was determined. At all time intervals studied, the concentration of radioactivity in the kidney in glucose-treated animals was less than in the controls. This was the only significant difference in the two groups. It was thought that glucose metabolism in muscle and liver and possibly other tissues, might have increased the demand for insulin in those tissues, and accordingly reduced the renal concentration. Possible increases in the concentrations of radioactivity in liver and muscle and changes in insulin breakdown rate were measured in a second experiment. Eight rats received a glucose load as above and eight control rats did not. All were sacrificed at 15 minutes and tissue radioactivity was divided into TCA precipitate and supernatant fractions. Changes were slight; a significant difference was not seen in any fraction.

Fructose was injected in similar fashion and concentration. The results are shown in Fig. 2. There was a significant reduction in both fractions of renal radioactivity, and a rise in precipitable radioactivity in liver and muscle. In all tissues there was less supernatant radioactivity. In an attempt to evaluate the effects of glucose and fructose on insulin metabolism in a more physiologic experiment, insulin-I^{131} degradation was measured during glucose and fructose tolerance tests. A procedure outlined by Lazarow(2) was used on rats which were fasted for 12 hours. A 10% aqueous solution of glucose or fructose was given intraperitoneally in a dosage of 3.5 g per kg of body weight, and 75 to 90 minutes later, insulin-I^{131} was given into the tail vein. Control rats were fasted 13 hours and received no intraperitoneal injection. All were sacrificed 15 minutes after insulin-I^{131}. The results are shown in Fig. 3. Glucose and fructose produced essen-

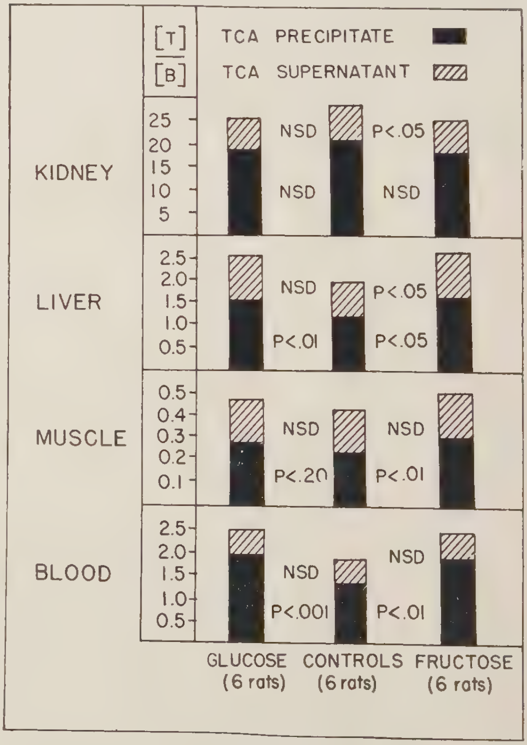

FIG. 3. Effects of intraperitoneal glucose, and fructose, on insulin-I^{131} degradation.

tially the same results. Significant changes were not produced in any supernatant fraction, however, precipitable radioactivity was increased in liver, muscle, and blood. Perhaps these changes were those anticipated above, namely, increased insulin concentration at sites of increased demand for insulin action. It is important to note that the changes were in the direction of *decreased* degradation, and not of *increased* degradation.

The effect of insulin deficiency on the distribution and breakdown of insulin-I^{131} was studied by means of *alloxan*. A dose of 40 mg/kg was given intravenously to fasted rats, and the insulin-I^{131} was given 24 hours later. All were glycosuric at this time. The animals had to be used shortly after alloxanization because their subsequent rapid weight loss would have made selection of control animals difficult. The results are shown in Fig. 4. Precipitable radioactivity was increased in blood and liver. Supernatant fractions were decreased in kidney and blood, and increased in liver.

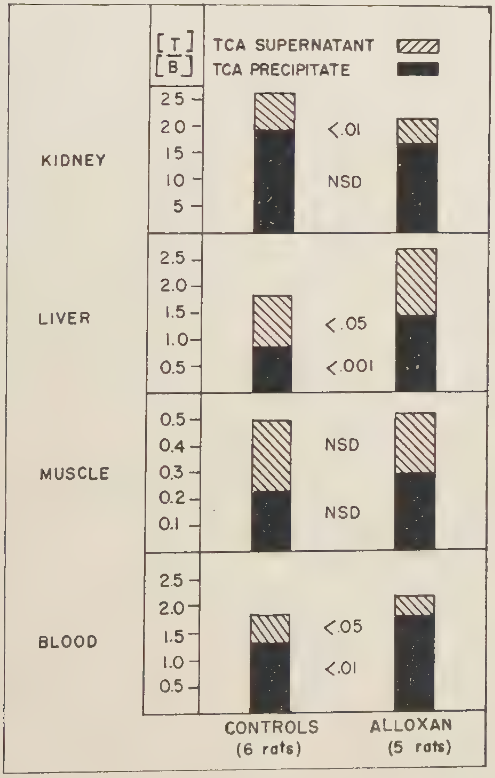

FIG. 4. Effect of alloxan on degradation of insulin-I^{131}.

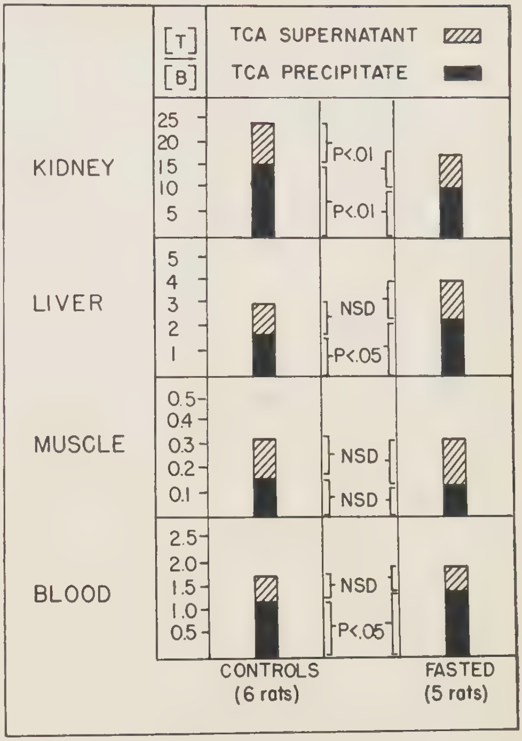

FIG. 5. Effect of fasting on degradation of insulin-I^{131}.

Phloridzin. Because of the remarkable renal concentration of precipitable radioactivity with insulin-I^{131} and the known inhibition of tubular glucose reabsorption by phloridzin, the effect of the latter on insulin-I^{131} metabolism was studied. Rats were each given 30 mg of phloridzin in 0.2 ml of propylene glycol subcutaneously. Five hours later they were glycosuric and insulin-I^{131} was given. The results showed that phloridzin was entirely without effect on the distribution and breakdown of insulin-I^{131}.

Fasting. Chiefly because of the possibility of the distribution being related to an insulin inactivating system(1) and the demonstration by Mirsky, *et al.*(3,4), of varying "insulinase" activity in rat liver after fasting, a group of rats was fasted 72 hours before receiving insulin-I^{131}. The results are shown in Fig. 5. Some increase in precipitable radioactivity in liver and blood and less radioactivity in kidney fractions were the noteworthy findings.

Discussion. It is of interest that in all instances where changes were effected in the kidney fractions they were in the direction of lowering the concentrations of radioactivity. The lability of this site has been noted before in our studies. Quite noteworthy however, was the lack of any change, even in renal radioactivity, with phloridzin. Insulin has been regarded as unrelated to renal tubular glucose reabsorption and this may support that concept. The data do not clarify the question of the function of the renal tubular insulin-I^{131}. Glucose did not produce any change, except some lowering of renal radioactivity when a hypertonic dose of 0.5 g was given intravenously over a short period of time. Degradation of insulin-I^{131} was apparently somewhat reduced at the height of an intraperitoneal glucose tolerance test. It is therefore of considerable interest that the receptors of insulin-I^{131} were apparently not greatly altered even in the face of large glucose loads, and that when insulin presumably was in increased demand and having accelerated physiologic activity, its degradation was not accelerated and possibly was reduced. It might also be considered that with insulin being used in glucose metabolism elsewhere, the renal insulin was reduced. These data are suggestive that the degradation and renal concentration of insulin-I^{131} are related to insulin inactivation rather than to insulin action. All of these effects were more pronounced with fructose, but the explanation for this is unknown.

Since with alloxan cellular damage is not restricted to the pancreatic beta cells, but occurs in kidney and liver as well, some of the changes seen with this drug may have been related directly to cellular damage. It is nevertheless significant that alterations in insulin-I^{131} metabolism were produced.

Mirsky has found insulinase activity to be decreased after fasting when he used liver homogenates(3), and to be increased when he used liver slices(4). Our studies show a decrease of precipitable radioactivity in kidney and an increase in liver. There was a slight, but not a clear cut, trend toward decreased degradation. How these studies interrelate with those of Mirsky remains to be elucidated.

There have been many undefined variables in these studies. Among them, the following should be mentioned—changes in fluid compartment volumes, changes in blood flow, exchange of precipitable and non-precipitable radioactivity between tissues and fluids, and redistribution of breakdown products. Further definition of breakdown products and correlation of such with function should offer a foothold into a better understanding of some of these variables and many of the results. Present work is being developed along these lines.

Summary. 1. The distribution and breakdown of insulin-I^{131} in rats under various changes in carbohydrate metabolism has been studied. 2. An intravenous glucose load exerted no detectable effect other than to reduce renal radioactivity. 3. Intravenous fructose markedly reduced renal precipitable radioactivity, and increased hepatic and muscle radioactivity. 4. Intraperitoneal glucose or fructose has the same effects as intravenous fructose but to a lesser degree. 5. Alloxan diabetic animals concentrated more precipitable radioactivity in the liver and blood. 6. Phloridzin exerted no effect on insulin distribution and degradation. 7. Fasting effected little change; there was some decrease in renal and some increase in hepatic precipitable radioactivity. 8. The limitations and implications of the study are discussed.

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Growth of Radiation-Induced Lymphoid Tumor Grafts in Hypophysectomized C57BL Mice.* (21327)

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Few reports have appeared on the growth of transplantable tumors in hypophysectomized animals. Ball and Samuels(1,2) were the first to show that the growth rates of 2 transplantable sarcomas were markedly slower in hypophysectomized rats than in intact controls. They were able to demonstrate that the retardation was due to the absence of pituitary secretions *per se*(3,4). Loefer(5) and Franseen and McTiernan(6) have also noted retardation of transplanted tumor growth in hypophysectomized rats. McEuen and Thomson(7) observed retardation of growth of transplanted Walker tumors in hypophysectomized rats; they concluded, however, that the slower growth was not due entirely to the absence of pituitary secretions, but in part to the dietary restrictions suffered by hypophysectomized animals. When hypophysectomized and intact rats were maintained on the same food intake, these investigators observed no difference in tumor growth between the 2 groups. Recently, Talalay and associates(8) have demonstrated that the growth of intramuscular implants of the Walker tumor was inhibited in hypophysectomized animals to about 46% of that in intact controls when both groups were tube fed and their body weights maintained at approximately the same level.

All of the above observations were made in the rat and the author is aware of only one other reported study carried out in mice(9). By grafting spontaneous mammary carcinomas obtained from dilute Brown-Murray-Little strain mice into hypophysectomized and intact groups of animals, Korteweg and Thomas(9) found no difference in tumor growth between the 2 groups. These investigators concluded that since the grafted tumors grew perfectly well in hypophysectomized mice, the influence of the pituitary is not a qualitative one, and if any influence exists, it can be only quantitative in nature. The present study was undertaken to determine whether radiation-induced lymphoid tumors of C57BL mice are capable of growing in hypophysectomized mice.

Materials and methods. One hypophysectomized and one intact group of mice were used in this experiment. Hypophysectomies were performed on 8 female and 6 male immature C57BL mice, 30 to 40 days of age and weighing 14 to 20 g, by the parapharyngeal method of Thomas(10) modified by the use of an Elliott corneal trephine to cut a window in the base of the skull. The animals were maintained in a warm room at 80 ± 2°F on a diet of bread soaked in Brewer's yeast-enriched milk,‡ lettuce, carrots, and Purina laboratory chow, and were given a 5% glucose and 0.9% sodium chloride solution as drinking fluid. Eight male and 8 female control animals were sham-operated and were also maintained on the above regimen except that tap water was given as drinking fluid. The experiment was started one month after the operative procedure, thus allowing sufficient time for the animals to recover. At this time the completeness of hypophysectomy was checked by comparative body weight determinations. All animals that became moribund or died were autopsied. Special attention was given to the target endocrine organs in the hypophysectomized animals. When lack of atrophy of these organs was indicated, the animals were eliminated from the final results. The radiation-

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‡ The proportions were as follows: 2.5 standard large loaves of white bread, 2 quarts homogenized milk, 50 g Brewer's yeast.

TABLE I. Growth of Radiation-Induced Lymphoid Tumor Transplants and Survival af Hypophysectomized and Intact C57BL Mice Bearing Tumor Grafts.

Group	No. of mice	Growth	Mean wt and range of tumor in g at death	Mean survival time and range in days after tumor transplant
Hypox. + tt*	14	13	1.0 (.7-2.0)	38 (14-49)
Sham + tt	16	14	1.5 (.9-2.3)	35 (24-39)

* tt = tumor transplant.

induced lymphoid tumor used in this study was obtained from the 69th transplant generation of a thymic lymphoma originating in a C57BL female mouse 203 days after the first of 12 daily exposures of 84 r each (1008 r total). The original transplant was made by Dr. Henry S. Kaplan in 1946 and has been successfully carried through subsequent generations in this laboratory. The tumor was excised and minced with a pair of fine scissors to provide small pieces which could be inserted into a No. 14 gauge trochar. One tumor fragment was implanted subcutaneously under the right axilla. Care was taken to use tissues of approximately the same size for each implant. Since these tumor implants grow diffusely and disseminate to all organs of the body rather quickly in intact animals (mean survival time after implantation is about one month), it was difficult to measure their rate of growth with precision during the course of the experiment. At weekly intervals, all animals were weighed and the growth of their implants was estimated by palpation. At autopsy the tumor was excised and weighed to the nearest 0.1 g.

Results. Results are presented in Table I. It may be seen that the radiation-induced lymphoid tumor grafts were able to take and to grow progressively in 13 of 14 hypophysectomized mice. However, analysis of the data[§] indicates that the growth rate of the tumor implants was significantly slower in the hypophysectomized group than in the controls during the first 4 weeks after implantation (Table II).

Mice from both groups began to die approximately 4 weeks after receiving the tumor grafts. One hypophysectomized mouse died 14 days after tumor transplantation and at autopsy no tumor was found. No implant was seen in one control mouse, although at autopsy the lymphoid organs were all greatly enlarged indicating dissemination of the tumor. One animal from the control group was sacrificed 8 weeks after the tumor was implanted; no remnant of the graft was found, and the animal appeared to be in good health.

The mean tumor weight in the hypophysectomized group was less (1.0 g) than in the control animals (1.5 g) at the time of death. No significance can be attached to these results since the tumors were not excised from all of the animals at the same time.

The mean survival time of the hypophysectomized mice bearing tumor grafts (38 days) was slightly longer than for the intact animals (35 days). The range was from 14 to 49 days and 24 to 39 days, respectively.

Discussion. Under the experimental conditions reported herein, it has been demonstrated that radiation-induced lymphoid tumor grafts are supported in hypophysectomized mice, and their ability to grow *per se* is not impaired by the absence of the pituitary. However, the rate of growth of the tumor transplants is significantly slower in hypophysectomized than in intact control animals. A longer period appears to be required after implantation of the tumors before any palpable growth can be observed in the hypophysectomized group.

It is well established that reduction of food intake may play an important role in the development and growth of spontaneous and transplanted tumors(7,8,11,12). It has been observed that hypophysectomized C57BL mice lose 10 to 20% of their body weight during the first month after operation, and subsequently fail to gain much beyond their initial body weight, while intact animals continue to grow (13). In view of this pronounced body weight difference, it appears likely that the slower growth rate of the transplanted tumors may be due to inanition, although this may not be the only factor responsible. Since the primary interest of this investigation was to ascertain whether hypophysectomized mice are capable

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[§] Analysis by the χ^2 test. $\chi^2 = 4.69$, $p<0.05$ at 28 days after tumor implantation.

TABLE II.
Estimation of Tumor Transplant Size in Hypophysectomized and Intact C57BL Mice.

Group	Total No. mice	No. mice showing 0 or + size growth* Days after transplantation 7	14	21	28	35	No. mice showing ++ to +++++ size growth* Days after transplantation 7	14	21	28	35	42
Hypox. + tt‡	14	14	14	11 (1)	9	2 (4)	0	0	2	3 (2)	7	6† (1)
Sham + tt	16	16	16	2 (1)	2 (1)	1	0	0	13	13	8 (7)	0 (15)

* 0 = no palpable growth; + = small palpable growth; ++ to +++++ = progressive increase in size of transplants.

† All remaining mice died by 49 days.

‡ tt = tumor transplant.

Numbers in parentheses indicate No. of mice dead.

of supporting radiation-induced lymphoid tumor grafts, the factor of restricted food intake was not considered. Hypophysectomy did not inhibit the development of radiation-induced lymphoid tumors in C57BL mice(14).

Recently Smith and coworkers(15) have shown that pituitary growth hormone appears to cause an acceleration in the growth rate of a transplanted mammary adenocarcinoma in C3H mice for periods up to 27 days. After this stage the effect seems to be reversed despite continued increase in somatic growth. This work suggests the possibility that the absence of growth hormone could also account in part for the initial retardation of tumor growth seen in the hypophysectomized mice of the present experiment.

Summary. Radiation-induced lymphoid tumor grafts were able to take and grow progressively in young hypophysectomized C57BL mice. The rate of tumor growth was significantly diminished in the hypophysectomized animals. Factors influencing tumor growth have been discussed.

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Intradermal Response to Egg Antigen in Humans with Active and Treated (Fuadin) *Schistosoma mansoni* Infections. (21328)

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Human infection with *Schistosoma mansoni* results in a dermal sensitivity so that skin reactions of the immediate or allergic type are elicited upon the inoculation of appropriate dilutions of antigens prepared from cercariae and adult stages (see review by Bozicevich and Hoyem) (1); studies carried on in this laboratory indicate stage specificities in the serological response to this parasite. No reports are available in the literature of the type of skin sensitivity which develops to an egg antigen nor of any temporal difference in its appearance. The following studies were undertaken to elucidate these questions.

Materials and methods. Preparation of skin test antigens. Eggs of *S. mansoni* were obtained from the livers of mice, experimentally infected with cercariae. About 8 weeks after exposure, the animals were sacrificed, the livers removed and comminuted in warm saline solution and adult worms removed. The suspension from about 3 livers was suspended in 50 ml of 1.7% sodium chloride to prevent hatching and ground in a Waring blendor. The liver tissue was removed by transferring the blended liver to a test tube held at an angle of about 15° on the stage of the stereoscopic microscope by a wedge of plasticine. In this position the eggs rapidly sedimented to the sides of the tube and the supernatant containing the liver debris was pipetted off. Fresh salt solution was added and the process repeated several times until no more extraneous material could be seen when examined at a magnification of 20x. The purified egg suspension was washed 3 times rapidly with distilled water in the lyophilization ampule, frozen in a dry ice-alcohol mixture, and lyophilized. Cercariae for the cercarial antigen were obtained from naturally infected snails and concentrated in conical sedimentation flasks at 6°C. The concentrated cercariae were transferred to lyophilization ampules, washed in distilled water, then shell frozen and lyophilized. In the final tests antigens were prepared by placing enough of the lyophilized material in 0.85% sodium chloride to make a 1% suspension. This was ground with a Tenbroek tissue grinder and then held at room temperature overnight with frequent shakings. It was then centrifuged at 3,000 rpm for 15 minutes. The clear supernatant was removed to constitute the 1:100 stock solution from which 1:10,000 dilution was made by adding appropriate volumes of 0.85% saline containing 0.5% phenol. Sterility tests in thioglycolate fluid medium and intracerebral inoculation in mice for toxicity were performed in all batches of antigen prior to use. *Test individuals.* The individuals tested consisted of 2 groups—21 adult continental Americans who had never been exposed to infection and 104 Puerto Ricans with a known history of long standing infection with *S. mansoni.* Thirty-six of these had never received drug therapy and were currently passing living ova, 33 had been treated with Fuadin within a year, and 35 had been treated from one to 20 years previously. *Skin test.* A 1:10,000 dilution of egg and of cercarial antigen as control was used. 0.05 ml was injected intradermally on the forearm by means of a 0.25 or 1 ml syringe and a 27 gauge needle. An injection of 0.05 ml of phenolized saline used for the antigen was given as a control. Dimensions of the wheal, presence of erythema and pseudopod formation were recorded immediately and 15 minutes after injection. A positive reaction in this study was considered to be one in which the sum of the diameters of the wheal at the 15 minutes reading was 4 mm or greater than the sum of the diameters of the original wheal.

Results. In these series of cases intradermal sensitivity was more frequent to cercarial an-

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TABLE I. Increase in Wheal Diameter and Presence of Erythema in Infected and Non-Infected Individuals 15 Minutes after Intradermal Injection of 1:10,000 Dilutions of Egg and Cercarial Antigens.

	Reactions to egg antigen				Reactions to cercarial antigen			
mm increase	No.	%	Erythema	%	No.	%	Erythema	%
			Infected individuals					
0	41	61.2	0	0	1	20.	0	0
1	12	17.9	0	0	1	20.	0	0
2	11	16.4	0	0	3	60.	0	0
3	3	4.5	0	0	0	0.	0	0
Total negatives	67	100.	0	0	5	100.	0	0
4 to 9	27	72.9	27	72.9	34	34.3	34	34.3
10 to 15	6	16.2	6	16.2	53	53.5	53	53.5
16 to 21	4	10.9	4	10.9	12	12.2	12	12.2
Total positives	37	100.	37	100.	99	100.	99	100.
			Non-infected individuals					
0	10	47.6	0	0	8	38.1	0	0
1	2	9.5	0	0	2	9.5	0	0
2	4	19.0	0	0	5	98.9	0	0
3	5	23.9	0	0	3	14.5	0	0
>3	0	0	0	0			0	0
Total negatives	21	100.	21	100.	21	100.	0	100.

tigen than to the egg antigen; 95.2% of the Puerto Rican subjects with a history of schistosomiasis gave positive reaction to cercarial antigen, but only 25.6% reacted to the egg antigen. The character of the reaction was similar to that given by cercarial and adult antigens with wheal and a flare appearing within a very short time after the injection. 72.9% of the reactions showed a 4 to 9 mm increase in wheal size (sum of 2 diameters) the remaining 27.1% showed an increase varying from 10 to 21 mm (Table I). Erythema occurred in all the reactions which showed an increase of 4 mm or more; pseudopodia occurred in 20.6% of the positive reactions. Among the continental Americans none showed erythema and in none did the wheal enlargement exceed 3 mm. The reaction to the cercarial antigen was somewhat more marked with 65.7% showing an increase in wheal size of 10 to 21 mm; all positive reactors showed erythema.

FIG. 1. Skin test reactions in patient with untreated *Schistosoma mansoni* infection, currently passing viable eggs in his stool. Egg antigen 1:10,000 upper right forearm, phenolized saline control below; adult antigen 1:5,000 upper left forearm, cercarial antigen 1:10,000 below.

The relatively large numbers of individuals with cercarial sensitivity but no egg sensitivity (Fig. 1) was found to lie in the group who had received no therapy for schistosomiasis. Only 8.3% of this group had dermal sensitivity to egg antigen while 91.7% reacted to the cercarial (Table II). In the group who had received therapy within the preceding year, a larger number (27.3%) acquired dermal sensitivity; in this group all reacted to cercarial antigen. In the group which had been treated more than one year before, 71.4% were sensitive to egg antigen (94.2% sensitive to cercarial) (Fig. 2).

Discussion. The presence of the same type of dermal sensitivity to egg antigen as to the cercarial or adult forms of the parasite was to be expected. However, the large number of individuals who were not sensitive to the egg antigen while markedly sensitive to cercarial antigen was an unexpected finding. This finding was an attribute of the actively infected individual (shedding viable ova at the time); after successful therapy and cessation

TABLE II. Intradermal Reactions to Egg and Cercarial Test Antigens.

Group description	Total No.	Positive reactions: Egg antigen 1:10,000 No.	%	Cercarial antigen 1:10,000 No.	%
A—No therapy	36	3	8.3	33	91.7
B—Therapy admin. within yr	33	9	27.3	33	100
C—Therapy admin. from >1 to many years previous	35	25	71.4	33	94.3
D—Non-infected individuals	21	0	0	0	0

of oviposition the prevalence of egg sensitization begins to approach that of cercarial sensitization. There would seem to be some process during active infection which inhibits the appearance of skin sensitivity. The constant outpouring of eggs by adult worms and the persistence of large numbers of living eggs in the tissues of the host may constitute a sufficient depot of antigen to neutralize the sensitizing antibodies before skin sensitization can develop. The appearance of such sensitization when therapy halts oviposition militates in favor of such a thesis.

The difference observed in the skin response to egg and cercarial antigens is more evidence to support stage specificity of antibodies appearing in infection with *Schistosoma mansoni.* Previous findings have indicated that anti-egg antibody in humans (circumoval precipitin) cannot be absorbed by treating serum with cercarial or adult lyophilized material(2). Here, in this study, there is clear cut immunological evidence of a special class of antibodies reacting with one phase of the parasite but not with the other.

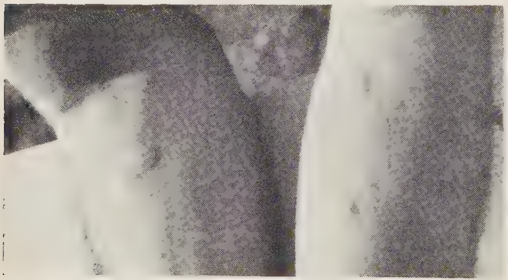

FIG. 2. Skin test reactions in patient with inactive schistosomiasis. No treatment given; last evidence of positivity was finding of one dead calcified egg in a 4 g fecal specimen one yr before skin test; complement fixation test weakly positive. Antigen sequence as in Fig. 1.

These findings suggest that such a dissociation of skin sensitivities indicates an individual who may be presumed to have an active infection, while the development of sensitivity to egg antigen denotes death of worms spontaneously or due to previous therapy, although not necessarily a total cure. Further work with a larger series of subjects is required to validate this assumption.

Summary. 104 Puerto Rican individuals known to be infected with *Schistosoma mansoni* were skin tested with antigen prepared from lyophilized eggs and cercariae. 35.6% reacted to egg antigen while 95.2% reacted to the cercarial antigen. The discrepancy was found to lie in those who were passing living eggs and had no skin sensitivity to egg antigen while those who had been treated more than a year before approached in percentage the skin reactivity with cercarial antigen.

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Elevation of Blood Magnesium and Potassium in Avian Erythromyeloblastic Leukosis.* (21329)

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The activity of the enzyme in the plasma of chicks with avian erythromyeloblastic leukosis dephosphorylating adenosine triphosphate(1-6) is greatly dependent on the influence of Ca^{++} and Mg^{++} in the presence of the monovalent ions Na^+ and K^+. These effects involving Ca^{++}, normally present in blood plasma, provoked an interest in possible variations of Ca^{++} as well as other ions, including Mg^{++}, K^+ and Na^+, in the plasma. Investigations revealed no change from the normal in Ca^{++} and Na^+ but striking increases in the concentrations of Mg^{++} and K^+. In addition it was evident that the levels of concentration of the latter ions were proportional to the capacity of the blood plasma to dephosphorylate adenosine triphosphate, and, consequently, to the concentration of virus particles in the plasma(2-4). The results of the studies are here described.

Materials and methods. The character of the disease investigated has been described in earlier reports(7,8). All of the studies were made on plasma obtained from individual White Leghorn chicks (Line 15)(9,10) which had been inoculated intravenously with the virus at 3 days of age. Onset of the disease was diagnosed by blood smears, and plasma donors were selected by the micro screening test(5) for enzyme activity. The series of plasmas studied was chosen to provide the broadest available range of activities of the plasmas based on adenosine triphosphatase activity. Blood was taken as usual, freed of cells by centrifugation, and the untreated and undiluted plasma was passed through celite #512 and through Selas filters(2). Studies on Ca^{++} and Mg^{++} were made with one series of plasmas and K^+ and Na^+ on another. Estimation of the dephosphorylating enzyme activity was made by electrometric titration(6) on 0.1 ml volumes of the plasmas in the presence of the saline solution balanced for the reaction. Calcium was determined by versene chelation with 0.5 to 1.0 ml plasma employing ammonium purpurate as the specific calcium indicator(11). Calcium solutions for standardization of the versene were prepared as described by Elliott(11). Magnesium was estimated by difference of the total versene chelation found with the indicator eriochrome black T, as described by Sobel and Hanok(12), and the amount of Ca^{++} obtained with ammonium purpurate. Sodium and potassium were measured by routine procedures with the flame photometer.†

Results. The findings observed with Mg^{++} and K^+ are summarized in Fig. 1. It is seen, in the abscissa of the figure, that the concentrations of K^+ varied from the normal low level of about 17-20 mg % to that of 34 mg %. A qualitatively similar elevation occurred with

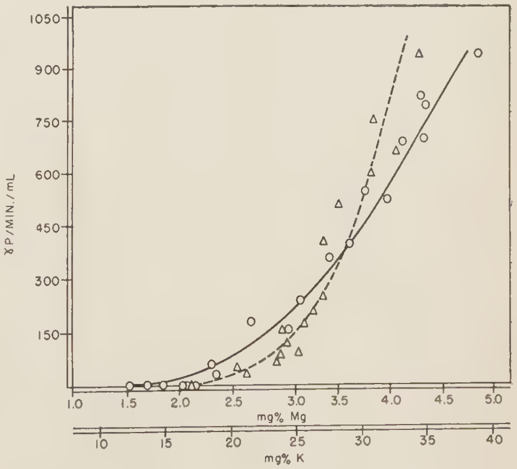

FIG. 1. Relation of ATPase activity and content of Mg (circles) and K (triangles) in plasmas of chicks with erythromyeloblastic leukosis.

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Mg^{++} from the range of 1.5 - 2.1 to 4.7 mg %. In those birds showing the elevations of Mg^{++}, the Ca^{++} values showed little, if any, change, varying only from 8.82 to 10.58 mg %; the same was seen with Na^{+}, varying from 359.3 to 374.4 mg % in the plasmas on which the K^{+} estimations were made.

In the same experiments, determinations were made of the activity of the respective plasmas to dephosphorylate adenosine triphosphate. It was observed that the increase in the levels of K^{+} and Mg^{++} in the plasmas was closely paralleled by increases in the enzyme content of the plasmas as clearly shown in Fig. 1.

Discussion. Our experiments have revealed relatively high concentrations of the normally intracellular ions Mg^{++} and K^{+} in the plasmas of birds with erythromyeloblastic leukosis without concurrent abnormality in the extracellular ions Na^{+} and Ca^{++}. It was observed also that the levels of concentration of these ions paralleled the ATPase activity of the plasmas. There is evidence(2-4) that the enzyme activity is a function of the virus of the disease and, consequently, the levels of Mg^{++} and K^{+} are likewise proportional to the virus content of the plasmas. Since virus does not multiply outside cells, it is evident that the agent bearing the enzyme activity must have been derived from the circulating primitive cells characteristic of the disease or from their diseased progenitors in the bone marrow or elsewhere. The mechanism of the accumulation of the ions in the plasma is more obscure. The possibility must be considered that the excessive amounts of Mg^{++} and K^{+} may reflect systemic effects of the disease involving tissues other than those immediately concerned with virus infection. In this respect there was no evidence of limitation in mechanisms of elimination since Ca^{++} and Na^{+} remained at normal concentrations. It would appear more likely that the Mg^{++} and K^{+} were derived from cells directly involved in the disease process, as in the case of the ATPase, or, possibly, from the red blood cells. There is some reason to suspect the red cells as contributors to the phenomenon, since with onset of the disease, a profound anemia develops(4). While this may be related in part to lessening of the rate of red cell formation, the rapidity and extent of the decline in red cell count is indicative of profound and progressive destruction of the red cells. Though this would appear sufficient to explain the findings with Mg^{++} and K^{+}, the great number of primitive cells(4) in the circulation constitutes, possibly, a more likely source of the ions. At the moment there is no direct evidence to suggest changes in membrane permeability or destruction of these cells which might liberate intracellular elements into the circulation. It is reasonable to suppose, however, that the mechanism by which virus is liberated might be responsible, likewise, for the findings with Mg^{++} and K^{+}. This is the more interesting in view of the close relationship between the concentrations of Mg^{++} and K^{+} and the levels of enzyme activity. The question is not subject to resolution at the present time, but the data provide an additional basis valuable for further investigations of host-virus relationships in erythromyeloblastosis.

Summary. Studies were made on the levels of Ca^{++}, Na^{+}, Mg^{++} and K^{+} in the plasma of birds with erythromyeloblastic leukosis. No changes from the normal were observed with the extracellular elements Ca^{++} and Na^{+}. In contrast, the ordinarily intracellular elements Mg^{++} and K^{+} were elevated in close relationship to the capacity of the plasmas to dephosphorylate adenosine triphosphate and, consequently, to the virus content of the plasmas. The source of the abnormal amounts of Mg^{++} and K^{+} have not been determined. The possible origin of the elements was discussed.

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Complement Fixation in the C-reactive Protein System. (21330)

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C-reactive protein (CRP) is a substance which appears in the blood as a result of varied pathologic processes(1-3). It is named for its capacity to form a precipitate with the somatic C-polysaccharide of the pneumococcus. CRP generally reaches a peak very early in the course of infectious diseases and then declines. Its presence appears to be associated with an inflammatory reaction. What its physiological role or function may be is not clear. Determination of C-reactive protein is used clinically as an index of rheumatic fever activity(4) and of myocardial necrosis and inflammation(5). In the rabbit, a positive correlation exists between a protein analogous to human CRP and the subsequent level of antibody production (6). An absolute quantitative method has been described for the estimation of CRP(7). Two additional methods have been used for CRP detection and relative estimation. The first of these involves the precipitation of the pneumococcal C substance, the somatic polysaccharide present in all types of pneumococci. A more sensitive method was later developed which depends upon the precipitation of CRP by rabbit antiserum to CRP. The latter method involves a true antigen-antibody reaction in contrast to the original method which does not.

This report will describe a complement (C′) fixation technic based on the reaction between CRP in human test sera functioning as antigen and rabbit CRP antisera. It is considerably more sensitive and precise than the precipitin method. The procedure set forth represents a modification of a C′ fixation method applicable to other immune systems developed by Osler, Strauss, and Mayer(8).

Materials and method. Human test sera. Test sera were inactivated at 56°C for 10 minutes. *CRP antisera.* Rabbit antisera prepared by Schieffelin and Co., New York, for the CRP precipitin test were used. The antisera were not heated prior to testing, since heating at 56°C for 10 minutes or longer diminished to a slight degree its complement-fixing potency and since its complement contribution was negligible. *Guinea pig C′.* Commercial lyophilized guinea pig C′ restored to its original volume with the diluent supplied by the manufacturer was used. The C′ amount was seven 50% units ($C'H_{50}$) contained in a total volume of 0.5 ml. *Sensitized sheep red blood cells.* A sheep red cell suspension (3%), sensitized with an equal volume of an optimal dilution of rabbit antisheep amboceptor(8), constituted the indicator system. *Diluent.* Veronal buffer, providing optimal concentrations of Ca and Mg(8), was the diluent for all reagents.

Procedure. The C′ fixation reaction was performed according to the outline in Table I. Constant quantities of C′, buffer, and rabbit CRP antiserum were added to 0.1 ml of varying dilutions of the human test sera. The test tubes were incubated at 5°C for 16 hours and sensitized sheep red cells were added. The tubes were then placed in a waterbath at 37°C for 30 minutes and the degree of hemolysis was estimated by color standards(8). The highest dilution in which appreciable fixation occurred (not more than 75% hemolysis) was considered the serum titer. A normal human

TABLE I. C′ Fixation Test for CRP.

Test tubes		Control tubes				
		Serum	C′	CRPA	Normal rabbit	Red cell
	ml					
Human serum dilution*	.10	.10	0	0	.10	0
7 $C'H_{50}$ in .5 ml	.50	.50	.10	.50	.50	0
Veronal buffer	.50	.60	1.10	.60	.50	1.20
CRP antiserum dilution†	.10	0	0	.10	0	0
Normal rabbit serum dilution	0	0	0	0	.10	0
	Fixation—16 hr at 5°C					
Sensitized sheep RBC, ml	.30	.30	.30	.30	.30	.30
	Hemolysis—30 min. at 37°C					

* Several 2-fold dilutions ranging from 1/1 to 1/512 or greater.
† 1/50 with one lot of antiserum.

control serum was similarly tested in each protocol. The normal rabbit serum dilution was the same as the CRP antiserum dilution. Fixation was not observed in the normal rabbit serum control. The serum, CRP antiserum, and normal rabbit control tubes should show complete hemolysis. The red blood cell control should show no hemolysis. The dilution of CRP rabbit antiserum which was used varied with its potency. One lot has been used at 1/50 dilution. The maximum quantity of CRP antiserum which may be used is restricted by the anticomplementary action of rabbit sera. Since strong complement fixing activity is evident far beyond the anticomplementary range, the test was standardized by a comparison of the reaction of different lots of antiserum against a reference human serum positive for CRP. *Precipitin test method.* The precipitin test was carried out in capillary tubes(9) employing a 2 cm long column of CRP antiserum and a column of test serum of equal length. The mixture was incubated for 2 hours at 37°C. The degree of precipitation (0 to 6+) was read after overnight refrigeration. Approximately 1 mm of precipitate was considered 1+; 2 mm 2+ and so forth.

Results. Parallel tests with the complement fixation and precipitation methods were performed with most sera. Greater sensitivity and precision were observed with the complement fixation method. For example, test sera giving a 1+ reaction in the precipitin test gave

TABLE II.
Comparison of the Precipitin and Complement Fixation Methods for CRP Determination.

	Precipitin	C.F.								
1. Epidemic hemorrhagic fever patient										
		Test serum dilution								
		1/1	1/5	1/25	1/125	1/625				
3 days after onset of illness	3+	0	0	3	4	3				
23 *Idem*	Neg	0	4	3	0	0				
2. Patient with generalized urticaria caused by penicillin therapy										
		Test serum dilution								
		1/2	1/4	1/8	1/16	1/52	1/64	1/128	1/256	1/512
3 days after onset of reaction	3+	0	0	0	0	4	4	4	3	2
6 *Idem*	1+	0	0	+	4	4	2	1	0	0
9 ”	Neg	0	±	∓	2	+	0	0	0	0
3. Individual inoculated with triple typhoid vaccine, 0.5 ml subcut.										
		Test serum dilution								
		1/1	1/5	1/25	1/125	1/625				
Pre-immunization specimen	Neg	0	0	0	0	0				
40 hr after immunization	1+	0	4	4	3	0				
7 days *Idem*	Neg	0	1	0	0	0				

0 = complete hemolysis; 4 = no hemolysis.

TABLE III. Reaction between CRP Positive Human Serum and Rabbit CRP Antiserum.

CRP antiserum dilution	Human serum dilution										
	1/5	1/10	1/20	1/40	1/80	1/160	1/300	1/640	1/1280	1/2560	1/5120
1/ 45	0	0	1	3	4	4	4	4	3	±	0
1/ 67.5	0	0	0	+	4	4	4	4	2	0	0
1/101.25	0	0	0	0	0	4	4	2	0	0	0

0 = complete hemolysis; 4 = no hemolysis.

complete fixation when 0.1 ml amounts of a 1/32 dilution were tested against 0.10 ml of a 1/50 dilution of a CRP antiserum. With a number of sera where the precipitin reaction was negative, marked reactions were obtained with complement fixation. The results with the complement fixation procedure have indicated that CRP persists for longer periods of time than less sensitive methods would indicate (Table II).

Sera from patients with a number of varied conditions were tested. Positive reactions were obtained with sera from patients with rheumatic fever, rheumatoid arthritis, syphilis, fevers of unknown origin and a generalized urticaria caused by penicillin therapy. CRP was detected in 2 patients with vivax malaria and 1 patient with amoebiasis, indicating that protozoal infections may be added to those conditions eliciting CRP. One infectious mononucleosis patient, and 12 epidemic hemorrhagic fever patients showed evidence of CRP.

A group of sera were tested by the C′ fixation technic alone. Reactions were obtained with 9 of a total of 13 sera from normal pregnant women in the latter half of their pregnancies. Caution, therefore, is indicated in the use of CRP as an index of rheumatic activity in pregnant women. In addition, CRP has been demonstrated by the complement fixation method in the serum of an individual 7 days after the injection of triple typhoid vaccine, 0.5 ml subcutaneously, and 7 days after a smallpox vaccination.

Twenty-six presumably normal control sera were tested by complement fixation and found to be without reactivity with 2 exceptions. On further investigation, it was found that the reacting sera were from 2 individuals, one suffering from a cold, and the other from a neck abscess.

It was noted in Table II that the fixation of C′ is inhibited by an excess of test serum containing CRP. This inhibitory or zonal effect is also shown in Table III which gives a typical result in testing varying dilutions of a CRP positive serum from a rheumatic fever patient against varying dilutions of a rabbit CRP antiserum. Normal human serum constituents are probably partly responsible for the zonal effect since the addition of normal serum to the test mixtures inhibits the C′ fixation reaction. The possibility also exists that the zonal effect is enhanced by an excess of antigen (CRP) as occurs in other systems(10,11).

Summary. A complement fixation method based on the reaction between CRP in human test sera and rabbit CRP antisera has been described. This technic provides a precise and reproducible estimate of the relative amounts of CRP in human serum. The procedure has greater sensitivity than the precipitin method and may be of greater value in following the efficacy of therapy.

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Plasma Volume, Bleeding and Clotting Time on Hypothermic Dogs. (21331)

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In the course of various physiological studies on hypothermic dogs, some observations on plasma volume, hematocrit, bleeding and clotting times have been made.

Method and material. Plasma volume was measured by the dilution technic of Gregerson (1) using a known amount of Evans Blue (T-1824). Hematocrit values were measured in Wintrobe tubes spun 30 minutes at 3,000 r.p.m. Duke's method(2) was used for bleeding time; the capillary tube method(3) using the under-surface of the tongue was used for clotting time. A total of 44 dogs weighing between 8 to 40 kg were anesthetized with Nembutal. The external jugular veins were cannulized for subsequent withdrawal of blood. Endotracheal intubation was done on all dogs. Plasma volume was determined on a control series of 12 dogs before and after a 60 minute interval—mixing time remaining 25 minutes. In a second series of 16 dogs, plasma volume was estimated before and after 60 minutes of hyperventilation with oxygen through a windshield wiper. In the last series of 26 dogs, plasma volume was estimated before and after they were cooled to 23° - 25° C. The time allowed for mixing was 25 minutes due to the possible slowing of circulation. Hematocrit, bleeding and clotting times were estimated with each plasma volume determination at the temperature at which the samples of blood were obtained, *i.e.,* 37°C with normothermic and 25° C with hypothermic animals.

Results. The table shows the changes in plasma, cell volume, bleeding and clotting time. Control series do not indicate any statistically significant change. The second series reveals an increase in all the elements of which plasma volume and clotting were significant. The third series shows a decrease in plasma and cell volume and an increase in bleeding and clotting time. All these values were significant.

Summary. An attempt has been made to show some changes under hypothermia with control studies on two factors, repeated plasma volume determination and hyperventilation. A moderate amount of blood seemed to be trapped away from circulation at lowered body temperature(4-6). Since blood clotting involves the action of enzymes it might be expected that coagulation would be retarded by lowered temperature. Increases in bleeding and clotting times at 23° - 25° C were highly significant.

TABLE I. Plasma and Cell Volume, Bleeding and Clotting Time.

		No. of dogs	Avg initial value	Avg value after 60 min.	Avg diff. of individual means ± SE
A Controls 60 min. interval	PV	12	55.1 *	55.3 *	+ .26 ± 1.59
	CV	12	42.8 *	46.0 *	+3.01 ± 2.04
	BT	11	1.20†	1.31†	+ .113 ± .203
	CT	11	2.15†	2.09†	− .068 ± .223
B Hyperventilation 60 min.	PV	16	54.5 *	60.2 *	+6.01 ± 1.97 ‡
	CV	16	42.9 *	47.4 *	+4.56 ± 2.29
	BT	16	1.20†	1.29†	+ .078 ± .139
	CT	16	1.85†	3.01†	+1.265 ± .386‡
C Hypothermia 23-25°C	PV	26	45.4 *	40.7 *	−4.44 ± 1.21 ‡
	CV	26	38.6 *	34.6 *	−3.94 ± 1.94 ‡
	BT	21	1.34†	3.02†	+1.630 ± .325‡
	CT	21	2.20†	4.10†	+1.888 ± .247‡

* ml/kg. † minutes. ‡ Significant—at the 1% level or better.
PV = plasma vol; CV = cell vol; BT = bleeding time; CT = clotting time.

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Influence of Vitamin B_{12} Upon Vaginal Patency in the Rat. (21332)

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Vaginal patency has frequently been taken as an indication of the onset of sexual maturity in the rat. It has been reported(1-3) that when rats weaned from mothers fed a B_{12}-deficient ration during lactation were continued on such a ration, the vaginas of those fed a supplement of vit. B_{12} or of B_{12}-active material opened at earlier ages than did those of their unsupplemented littermates. The present paper is concerned with further and more complete data bearing on this question.

Procedure. Except where otherwise indicated, the rats used in these experiments were weaned from stock colony mothers that had been transferred at parturition from a natural food ration(4) to a semi-purified B_{12}-deficient ration and maintained on this deficient ration during the ensuing lactation. During the experimental period, the animals were housed in individual cages furnished with raised screen floors. All comparisons have been made between pairs of littermate rats. The principal B_{12}-deficient rations fed to the experimental animals were rations 45 (groups 1-3, Table I) and 262 (groups 4c,5); the ones fed to their mothers were rations 47 (groups 1-3) and 260 (groups 4,5). Ration 45 consisted of 55.5% dextrin, 20% 10x-hot-alcohol-extracted casein(4), 10% dried brewers' yeast, 4.5% salts(5), 9.85% cottonseed oil and 0.15% fish liver oil (containing, per gram, 65,000 I.U. of vit. A and 13,000 I.U. of vit. D). Ration 262 was similar to ration 45 except that 15% lactose replaced 15% dextrin and crystalline vitamins[†] were added. Ration 47 was similar to ration 45 except that the dextrin was decreased to 45.5% and the yeast raised to 20%. Ration 260 differed from ration 47 only in that 15% lactose replaced 15% dextrin. In groups 4 a-b, d-g, the other carbohydrates were substituted in ration 262 in place of the lactose-dextrin mixture to give a total of 55.4% of the carbohydrate indicated; soy protein, where used, replaced all the casein in the diet and part of the carbohydrate (dextrin in the case of the dextrin-lactose mixture) to give a total of 24.5% of this product ($\approx$20% protein). Except as otherwise indicated, the rats were fed *ad libitum.* Supplements of crystalline vit. B_{12}[‡] or APA liver extract, where not included in the ration, were administered orally by syringe 3 or 5 times weekly.

Results. The effect on vaginal patency of feeding a supplement of vit. B_{12} to young rats maintained on certain B_{12}-deficient rations is shown in Table I. Where the mothers were fed a B_{12}-deficient ration during lactation (all groups except 2b,3e,3f), the vaginas of the

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[†] Mg/100 g ration: thiamin hydrochloride, 0.5 riboflavin, 0.5; pyridoxine hydrochloride, 0.5; calcium pantothenate, 3.1; choline chloride, 75.; nicotinic acid, 3.1; inositol, 3.1; para aminobenzoic acid, 18.8; biotin, 0.006; pteroylglutamic acid, 0.06; ascorbic acid, 3.08; alpha-tocopherol acetate, 6.25; 2-methyl,1,4-naphthoquinone, 0.16. The pteroylglutamic acid was kindly supplied by Dr. E. L. R. Stokstad and Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

[‡] Kindly supplied by Dr. D. F. Green and Dr. M. A. Schooley, Merck and Co., Rahway, N. J.

TABLE I. Effect of Vitamin B_{12} Deficiency upon Vaginal Patency in Rats.

Group	No. of pairs of rats	Age at start of exp.‡	Source of carbohydrate in ration	Type of protein in ration	Age at vaginal patency§ No B_{12}	Age at vaginal patency§ B_{12}‖	Age at vaginal patency§ t	Body wt at vaginal patency§ No B_{12}	Body wt at vaginal patency§ B_{12}‖	Body wt at vaginal patency§ t	Wt gain during 1st wk of exp. No B_{12}	Wt gain during 1st wk of exp. B_{12}‖	Wt gain during 1st wk of exp. t
		(days)			(days)	(days)		(g)	(g)		(g)	(g)	
1 a	29	20–23	dextrin	casein	54.9	34.4	8.4†	97.5	90.4	2.5*	10	22	14.9†
b	23	28–29	”	”	48.2	37.5	5.2†	107.3	96.5	2.7*	19	30	6.8†
c	19	30–37	”	”	47.5	42.9	1.8	99.4	96.6	.9	17	27	5.7†
2 a	6	28	dextrin	casein	57.2	40.3	4.2†	114.0	104.0	1.3	18	30	5.2†
b¶	6	28	”	”	38.2	37.2	.3	106.7	102.0	.4	28	28	.1
3**a	13	28–29	dextrin	casein	47.5	35.6	3.3†	104.7	89.5	2.3*	17	27	7.0†
b	19	27–28	”	”	51.3	36.1	5.2†	101.9	85.6	3.8†	17	28	8.3†
c	24	27–29	”	”	48.5+	38.7	5.5†	94.5+	84.8	3.1†	16	28	8.2†
d	6	27–29	”	”	43.8+	35.0	3.4*	96.8+	81.3	2.4	16	28	3.2*
e¶	36	27–29	”	”	35.4+	34.6	1.1	91.7+	88.2	1.6	25	28	3.4†
f¶	15	27–28	”	”	34.8	35.9	.9	88.0	89.4	.4	25	23	.7
4††a	8	20–22	corn meal	casein	46.5	35.6	5.4†	107.4	88.9	3.4*	15	22	3.1*
b	13	20–22	cornstarch	”	47.2	34.7	3.6†	111.5	97.5	2.7*	21	28	3.8†
c‡‡,§§	60	19–23	lactose-dextrin	”	41.1	31.7	8.5†	78.2	78.0	.1	15	24	15.5†
d	8	20–22	sucrose	”	42.0	34.5	3.2*	102.8	90.5	1.8	21	27	4.2†
e	7	20–23	corn meal	soy protein‖‖	57.1	34.3	7.2†	91.7	82.7	.8	12	25	5.7†
f	16	20–23	lactose-dextrin	*Idem*	34.4	29.6	3.8†	50.9	60.4	2.9*	8	21	18.0†
g	8	20–23	sucrose	”	63.1	33.2	4.8†	79.9	80.4	.8	9	24	10.4†
5¶¶	10	20–22	lactose-dextrin	casein	44.8+	36.8	4.6†	94.3+	75.8	4.2†	14	12	.4

* and † See text footnote §.

‡ At these ages, rations and supplements were first fed; previously, rats had access to maternal rations. Where age is 23 days or less, weaning occurred simultaneously with start of experiment; where age is greater than 23 days, weaning occurred at approximately 25 days of age.

§ In a few cases, vaginas of rats in the "no B_{12}" groups were not open at the time of discarding; these rats, however, have been included in the averages with ages and body weights as of that time. These cases consisted of 7 rats in group 3c and 1 each in 3d and 3e (56–63 days of age) and 4 in group 5 (49 days of age).

‖ Groups 1, 2 and 3—Lederle 15U/ml APA liver extract (0.02-0.05 ml/day); group 4—crystalline vit. B_{12} (2 μg/day, 0.5 μg/10 g ration or 1 μg/10 g ration); group 5—crystalline vit. B_{12} (2 μg/day).

¶ Mothers of these rats fed either 0.05 ml (3e, 3f) or 0.10 ml (2b)/day Lederle 15U/ml APA liver extract.

** Group 3 consists of rats maintained on experimental rations over several generations: a, Parent generation; b and e, F_1 generation; c and f, F_2 generation; d, F_3 generation. See (6-8) for details.

†† F values for effect of type of carbohydrate and protein upon differences between no B_{12} and B_{12} (comparing groups a, c-g): age at vaginal patency—carbohydrate, 12.9†; protein, 9.8†. Body wt at vaginal patency—carbohydrate, 4.9†; protein, not significant (mean square less than error mean square). Wt gain during 1st wk of experiment—carbohydrate, not significant (mean square less than error mean square); protein, 30.3†.

‡‡ Averages for B_{12} rats in 10 pairs were derived from avg of 2 rats in each litter, one receiving crystalline B_{12} (2 μg/day) and the other APA liver extract (0.10 ml/day). No significant difference between liver extract and B_{12} groups in this experiment was observed either in age ($t = 1.1$) or in body wt ($t = 1.2$) at time of opening or in wt gain during first wk on experiment ($t = 1.2$).

§§ For 10 pairs, wt at time of vaginal patency: ovaries (no B_{12}), 28.5 mg; B_{12}, 35.1 mg; $t = 1.9$; uterus (no B_{12}), 41.2 mg; B_{12}, 104.3 mg; $t = 4.5$†.

‖‖ Drackett Soybean Protein #220 (Drackett Co., Cincinnati, O.).

¶¶ Pair fed.

B_{12}-supplemented young opened on the average at earlier ages in all groups and at smaller body weights in most groups. It is evident that in such groups, when *ad lib.* fed, this earlier vaginal patency was accompanied by a more rapid growth rate. The differences in vaginal patency between B_{12}-supplemented and B_{12}-deficient rats (group 1) were significant§ when the supplements were started at 20-29 days of age but not when they were initiated at later ages.

When vit. B_{12} was fed to the mothers during lactation, no difference in age of vaginal patency was observed (group 2b compared with their littermates in group 2a which were nursed to mothers on B_{12}-deficient rations; see also groups 3e and 3f). Continuing animals on B_{12}-deficient rations over several generations (group 3) did not seem to increase the differences in either vaginal opening age or weight between B_{12}-deficient and B_{12}-supplemented young. Thus the results obtained in groups 3a, 3b, 3c and 3d are about the same even though each succeeding subgroup represents an additional generation on the B_{12}-deficient ration.

The series of experiments shown in group 4 were designed to study the effect of altering the type of carbohydrate and protein in the ration on the relation between vit. B_{12} and vaginal patency. With animals fed casein rations, the differences in age at vaginal patency between B_{12}-deficient and B_{12}-supplemented young were about the same regardless of the carbohydrate source. When soy protein was used as the protein source, however, these differences were much greater with either corn meal or sucrose than with a lactose-dextrin mixture. The body weights of the B_{12}-deficient females at time of vaginal patency were greater than those of the B_{12}-supplemented animals only with casein rations and then only with certain carbohydrates. Somewhat greater differences (between B_{12}-deficient and B_{12}-supplemented rats) in growth rates during the first week on experiment were observed with the soy protein rations than with the casein rations in group 4. The results obtained by altering the carbohydrate (and perhaps also the protein) may possibly be attributed to the effects of these substances on intestinal synthesis in the animal.

Although with the lactose-dextrin casein ration, with *ad lib* feeding, no difference in body weight at time of opening was observed, there is some evidence indicating more advanced sexual development of the B_{12}-supplemented young at this time. In one group (5, Table I) in which the food consumption of the B_{12}-supplemented young was limited to that of the B_{12}-deficient young, and, as a consequence, the rate of weight gain of both groups was essentially the same, both vaginal opening age and body weight at the time of vaginal opening were higher for the B_{12}-deficient young. Furthermore, in an *ad lib* fed group on this ration (footnote §§, Table I), in which no significant difference in body weight at time of vaginal opening was observed, the B_{12}-supplemented young had at this time significantly higher uterine weights and somewhat, although not quite significantly, higher ovarian weights than the B_{12}-deficient young.

Summary. The onset of sexual maturity in female rats, as measured by vaginal patency, was found to be delayed in vit. B_{12}-deficient animals as compared to vit. B_{12}-supplemented littermates when their mothers were fed a B_{12}-deficient ration during lactation. No such differences were observed when the mothers were fed B_{12} during lactation. The nature of the carbohydrate and protein in the ration was found to have an effect on the differences observed.

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Received August 2, 1954. P.S.E.B.M., 1954, v87.

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§ The symbol † adjacent to a *t* or *F* value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no * indicates no statistically significant difference.

Comparative Activity of Pantothenic Acid and its Conjugates for Tetrahymenid Ciliates.* (21333)

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It has been demonstrated(1) that pantothenic acid (PA)[†] is essential for the growth of the ciliate *Tetrahymena pyriformis* W. Investigation of the requirements of *Glaucoma scintillans* and *Colpidium campylum*(2), closely related organisms, showed that much higher levels of PA are needed for growth.

Conjugated forms of PA have been shown to be more active than is the free acid for the growth of certain bacteria. *Lactobacillus bulgaricus* requires 75 to 500 times as much PA as it does pantetheine (LBF)(3,4), while for *Acetobacter suboxydans* 4′-phosphopantetheine (LBF phosphate) is 10 to 20 times as active as PA(5). It was therefore considered possible that these ciliates might respond more readily to conjugates than to PA.

Material and methods. Tetrahymena pyriformis W was cultured in medium A with Tween 80(6) in which the PA was replaced by various of its derivatives.

The media used for the growth of *Glaucoma scintillans* A and *Colpidium campylum* C were those already described(2). In the case of *Glaucoma,* either Difco Proteose-Peptone, Labco Casein or Armour's Bovine Serum Albumin could be used as a nitrogen source, since none of them contains an appreciable amount of PA. *Colpidium* was cultivated in the casein medium. All experiments were conducted using the methods previously described(2,6). The following compounds were tested for their ability to promote the growth of the ciliates: calcium pantothenate (PA), pantethine[‡] (LBF), dl-barium – 4′ – phosphopantetheine[§] (LBF phosphate), Coenzyme A[§] (CoA), dl-pantoyllactone and pantothenol.

Results. Pantothenol and pantoyllactone either with or without the addition of β-alanine were inactive for all of the 3 ciliates even at very high levels. As may be seen from Table I, PA is 5 to 7 times more active for *Tetrahymena* than is any of the conjugated forms of the vitamin. For Glaucoma, on the other hand, LBF is the most active form tested, but in all cases the requirements are larger than those of *Tetrahymena.* Essentially the same results are obtained whether proteose-peptone, casein or albumin is used in the medium. The most active compound for *Colpidium* is PA.

In the case of *Glaucoma,* this ciliate fails to respond to low levels of the vitamin. Growth occurs only in the presence of amounts greater than a certain "threshold" concentration. Attempts to eliminate this "lag" in the response by altering the composition of the medium or by prolonging the growth period were not successful. Addition of cysteine to the medium (5) had no effect on the response. The "lag" is also present when the coenzyme form of the vitamin is used. It therefore appears improbable that it is due to the need for conversion of the free acid to CoA.

It was reported earlier(7) that *Tetrahymena* is unable to utilize CoA for growth. At that time CoA was tested only at levels corresponding to the PA requirement of Tetrahymena. Such concentrations would be too small to produce a response, since *Tetrahymena* is capable of responding only to levels of CoA above 1 γ/ml. The usual explanation for the poor utilization of the coenzyme forms of a vitamin is that phosphorylated compounds are not readily assimilated. This hypothesis would account for the relative inactivity of LBF for *Tetrahymena* only if one assumes

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* This investigation was supported by a research grant (G-3866) from the National Institutes of Health, Public Health Service.

[†] The following abbreviations will be used: Pantothenic acid = PA; Panteth(e)ine = LBF; 4′–phosphopanteth(e)ine = LBF phosphate; Coenzyme A = CoA.

[‡] Kindly furnished by Dr. O. D. Bird, Parke-Davis Co.

[§] Kindly furnished by Dr. G. D. Novelli, Western Reserve University.

TABLE I. Activity of Pantothenic Acid Derivatives for Tetrahymenid Ciliates. Amount required for half-maximal growth, $\mu M \times 10^6$/ml.

	Ca pantothenate (PA)	Pantethine (LBF)	dl-4′-phospho-pantetheine (LFB phosphate)	Coenzyme A (CoA)
T. pyriformis	50	250	350*	400†
G. scintillans	900	400	850	950
C. campylum	400	1100	2750	2650

* Data corrected for 50% activity of dl-form.
† " " " 90% purity.

wide variations in permeability of the cells of the different species of ciliates. It cannot account for the differences in activity of the various forms of PA conjugates.

The amount of PA required for half-maximal growth of *Tetrahymena* is in the same range as that required by *Lactobacillus arabinosus*. It must therefore be assumed that the larger requirements of *Glaucoma* and *Colpidium* are deviations from the normal. As yet, no satisfactory explanation for this large demand is at hand.

Summary. Pantothenic acid is more active for growth promotion in *Tetrahymena* and *Colpidium* than are any of the conjugated forms of the vitamin. For *Glaucoma* pantethine is the most active form.

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Single Shock Conduction Pathways from the Preoptic Shivering Suppression Center.* (21334)

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It has been found recently that shivering in anesthetized animals can be suddenly inhibited by electrical stimulation of points on the cerebral cortex(1) or in the hypothalamus(2). The points of the hypothalamus which when stimulated suppress shivering extend throughout the hypothalamus and caudally into the midbrain, with, however, a particularly sensitive region being located in the preoptic and supraoptic portion of the hypothalamus. Due to the wide distribution of the suppressor points it is of interest to know if the active preoptic suppressor region is connected by nervous conducting pathways with more caudally located suppressor points. To investigate this problem points in the preoptic shivering suppressor region have been stimulated with single shocks and the electrical activity associated with the single shock recorded from points in the midbrain and posterior hypothalamus.

Procedure. Eight cats were anesthetized with pentobarbital sodium intraperitoneally (30 mg/kg), the brain was exposed from above and concentric needle electrodes were introduced into the brain stem by means of the

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* This work was supported in part by the U. S. Air Force under Contract, monitored by Alaskan Air Command Arctic Aeromedical Laboratory.

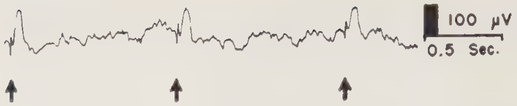

FIG. 1. Record of electrical activity from 2 pairs of needle electrodes placed in midbrain of a cat (coordinates A2, H-5, L3) after stimulating the preoptic shivering suppressor region with single shocks at time shown by arrows. An action potential wave as shown is designated a + response indicating a conducting pathway between stimulating and recording electrodes.

Horsley-Clarke stereotaxic apparatus. One of the electrode pairs was placed in the preoptic region or anterior hypothalamus for stimulating, and 2 or 3 pairs of recording electrodes were placed in a more caudal level. After vigorous shivering had begun, a stimulus of 2.5 volts at a frequency of 200 cps was applied by a condenser discharge stimulator. The position of the stimulating electrode was varied until a maximal shivering suppressor response was obtained. This setting was then used during the experiment as the site of stimulation. Its position varied from A19 to A14 (Horsley-Clarke coordinates) in individual cats. A 6-channel EEG instrument was used to record brain potentials and muscle potentials. The latter were obtained from bipolar skin electrodes applied over the biceps femoris muscle of the hind leg. Single shocks of 3 volts and 1 millisecond duration were sent into the suppressor region at a rate of 1 per second and the presence or absence of a conducted impulse was noted at each position of the recording electrodes. The series of experiments included records from 11 A-P settings (A6 to P9), making a total of 243 points tested. At 18 of the positions which gave a positive response the recording site was stimulated with 2.5 volts at 200 cps in order to test for shivering inhibition. Fifteen negative sites were stimulated in control experiments. Stimulation and recording points were located on histological slides of the sectioned brain which had been fixed in 10% formalin and stained with buffered thionin.

Results. An example of a positive response is shown in Fig. 1. The conducted impulse from the preoptic region can be distinguished from the background of spontaneous activity as a broad spike of about 100 micro V amplitude preceded by a shock artifact. This record was obtained from the upper midbrain tegmentum, in the region of the substantia nigra. Figure 2A shows location of the stimulating electrode in one such experiment. It is located below the anterior commissure in the preoptic region just rostral to the hypothalamus.

The pathway traced by this technique is diagrammed in Fig. 2 (B to D) which shows 3 representative cross-sections of the brain stem, B through the midbrain at the level of the superior colliculus, C through the pons and D through the upper medulla. These are composite sections and include the data obtained on all 8 cats. The conducting pathway from the shivering suppressor region was found to lie in the medial reticular formation of the midbrain, pons and medulla, in the substantia nigra and the ventral tegmentum of midbrain and pons. In the medulla the pathway assumed a more diffuse course in the reticular formation.

When a positive response was obtained, stimulation with the recording electrode (2.5V

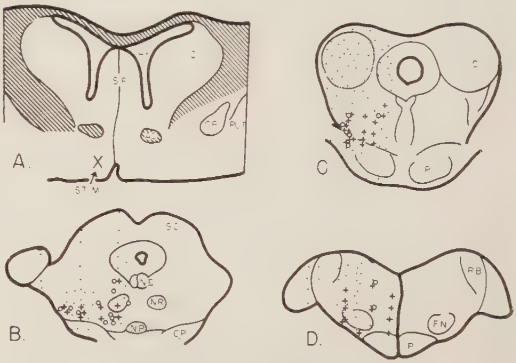

FIG. 2A. Point of electrical stimulation in preoptic region of cat brain (X). Stimulation of this point produced immediate cessation of shivering and + response of the conducted action potential to the points marked + in Fig. 2 B-D.

FIG. 2 B-D. Points in brain stem from which electrical records were made after stimulating preoptic suppressor region, shown in Fig. 2A, with single shocks. Action potential recorded after shock designated +, no action potential wave after shock designated •, shivering suppression when electrically stimulated designated ○. B—cross-section through superior colliculus, C—pons, D—upper medulla. AC—anterior commissure, C—caudate nucleus, CP—cerebral peduncle, FN—facial nucleus, IC—inferior colliculus, LV—lateral ventricle, NIII—oculomotor nucleus, NIP—interpeduncular nucleus, NR—red nucleus, P—pyramidal tract, PUT—putamen, RB—restiform body, SC—superior colliculus, SP—septum pellucidum.

at 200 cps) produced a lasting suppression of shivering in the 18 cases in which this test was made. These suppression points are indicated by circles on the diagrams in Fig. 2. As a control, 15 conduction-negative points were stimulated and in each case failed to suppress shivering. It was incidentally noted at 14 settings which yielded positive conduction and suppression that the insertion of the electrode caused a transient suppression of shivering by mechanical stimulation. This suppression lasted from a few seconds to a half hour.

Conclusion. There is a descending conduction pathway from the shivering suppression "center" in the diencephalon extending caudally through the medulla. Electrical stimulation along this pathway suppresses shivering. One interpretation of these results might be that central control of shivering is accomplished over this route. Instead of acting on the site of origin of the shivering impulses (presumably the posterior hypothalamus), the inhibitory influence may be exerted at lower motor centers, perhaps at the ventral horn outflow, although the conduction pathway in the cord has not been investigated. Direct suppression of the ventral horn cells innervating particular muscle groups would be a means of obtaining the graded response that is characteristic of the shivering process.

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Inhibition of Pituitary Gonadotropin Secretion in Domestic Fowl by Enheptin (2-Amino, 5-Nitrothiazole) (21335)

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The influence of pituitary hormone secretions may be altered by surgical ablation(1), by use of androgens(2) and estrogens(3). The most serious objection in the use of sex hormones to suppress the influence of pituitary gonadotropins lies in the development of side effects(4). Extracts of the plant Lithospermum ruderale have been reported to affect the sex cycle of mice and rats(5) and to reduce the incidence of mammary gland tumors(6,7) presumably through pituitary suppression (8,9). Reports of the supposed pituitary inhibiting action of parahydroxypropiophenone (PHP)(10,11) have been disputed(12,13). Recent reports from this laboratory have shown that the drug Enheptin† (2-amino, 5-nitrothiazole) caused complete suppression of sexual manifestations and functions in White Leghorn chickens(14,15). In turkeys (16), Enheptin at 0.1% dietary level reduced the level of reproductive performance. This

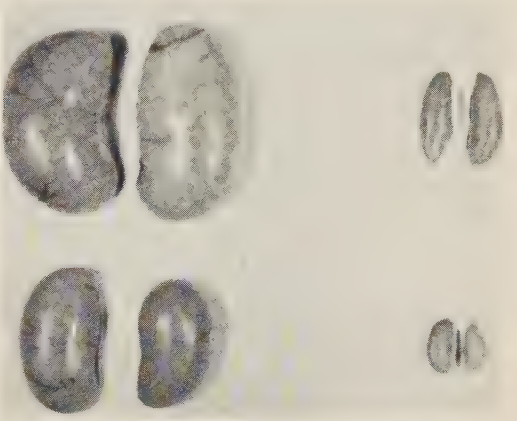

FIG. 1. Testicular atrophy in adult cockerels following oral administration of Enheptin at dietary level of 0.05% for 6 wk. Three-year-old control, upper left; treated, upper right. One-year-old control, lower left; treated, lower right.

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† Enheptin is a commercial product of the Lederle Laboratories, Amer. Cyanamid Co., used for treatment of infectious enterohepatitis.

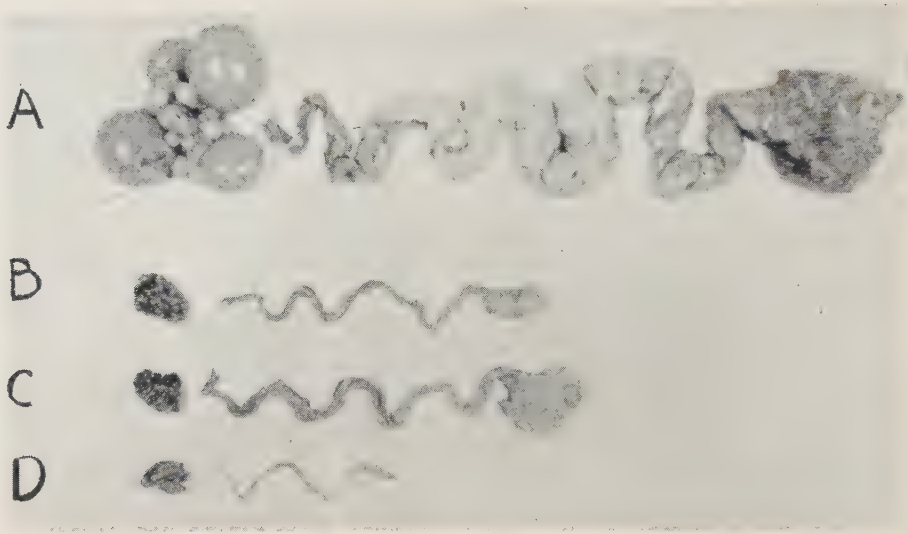

FIG. 2. Atrophy of ovary and oviduct following oral administration of Enheptin. A. Functioning ovary and oviduct of a hen. B. Nonfunctioning ovary and oviduct from healthy hen. C. Ovary and oviduct from hen treated with Enheptin for 4 wk at dietary level of 0.05%. This hen was laying at rate of approximately 67% at start of treatment. D. Ovary and oviduct from hen on continuous 0.05% dietary level of Enheptin for 13 mo and never laid an egg.

report presents observations which provide some evidence on the nature of action of Enheptin in suppressing sexual activity in White Leghorn chickens.

Procedure. The same general method of management was followed. All experimental birds were confined in cages. They were White Leghorns from a mass-mated, closed flock of the Experiment Station. Immature birds were kept in grower batteries, while adults were kept in individual laying cages. All immature animals were fed an all-mash grower diet designated N. J. No. 422, the ingredients of which were listed by Hudson and Pino(15). Adults were fed an all-mash laying ration designated N. J. No. 11. Except when indicated, all chickens were fed and watered *ad libitum*. Enheptin was administered orally as part of the diet. Artificial lights were provided daily from 4 to 8 a.m. Further details are presented with appropriate sections.

Results. Within a week following daily oral administration of Enheptin, there appear typical symptoms of sexual disfunction which grow in intensity for about 3 weeks when complete sexual suppression is achieved. The comb, one of the first external signs of gonadal involution, begins to shrink and shrivel until it becomes almost capon size. The shanks appear dehydrated. Molting is practically continuous. In the male, crowing ceases, copulation does not occur and aggressiveness is lost. In the laying female, egg production ceases within 7 to 10 days. The effect upon reproductive system is rather dramatic, especially in the adult (Fig. 1 and 2). Young chicks 3 to 8 weeks old, when given oral doses of Enheptin in sufficient amounts to make suppression obvious, will, if treatment is discontinued, make rapid recovery so that at the age of normal sexual maturity the previous sexual inhibition is unrecognizable (unpublished data).

TABLE I. Effect of Substitution Therapy with Exogenous Gonadotropins and Testosterone Propionate in Counteracting the Sexual Suppression Effects of Enheptin, 6 Groups.

		Testis size, mg/kg body wt		
No. of birds	Treatment*	End 15th wk	End 16th wk	End 17th wk
3	C†	632.4	540.0	4740
3	E + 50 IU G	562.9	321.6	280
3	E + 100 IU G	942.3	268.4	310
3	E	208.2	182.6	150
4	E + 2.5 mg T	198.2	—	—
4	E + 5 mg T	242.5	—	—

* Treatment began at 12 and terminated at 15 wk of age.

† E—Enheptin; G—Gonadin every other day; T—testosterone propionate every other day.

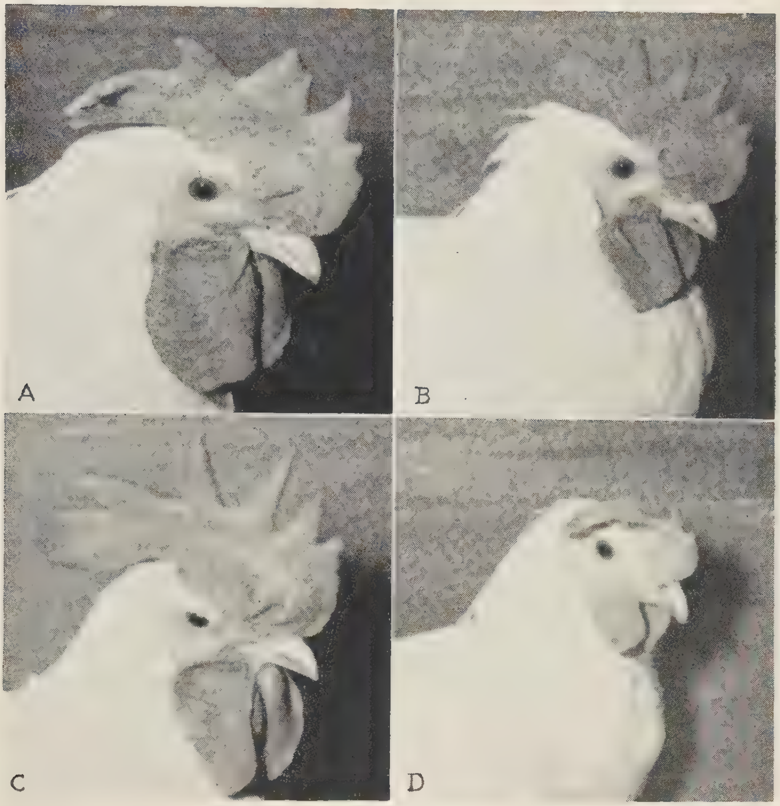

FIG. 3. Effect of gonadotropins in alleviating sexual inhibition. Appearance of cockerel at termination of 3-wk treatment period. A) Untreated cockerel. B) Enheptin fed plus 50 units Gonadin every other day. C) Enheptin fed plus 100 units of Gonadin every other day. D) Enheptin fed.

Effect of substitution therapy. One of the first objectives was to determine whether or not sexual disfunction precipitated by Enheptin, could be alleviated by exogenously administered gonadotropins. The inexpensive and readily available pregnant mare serum was used. In the first experiment, 12-week White Leghorn cockerels were placed on treatment, as shown in Table I. Enheptin was given at a dietary concentration of 0.05% for 3 weeks. During the same period hormone therapy was instituted as indicated. Drug and hormone were discontinued at end of third week of treatment. At the end of 15th, 16th, and 17th week, one bird from Groups 1 to 4 was killed and organ weights were taken. All birds in Groups 5 and 6 were slaughtered at the end of 15th week. Testis weights are shown in Table I.

The appearance of cockerels in Groups 1 to 4 at the end of 15th week is shown in Fig. 3a, b, c, and d, respectively. Testis sections are shown in Fig. 4. Although the cockerels receiving testosterone looked and behaved quite masculine and had well developed genital papillae, their testes showed histologically complete absence of spermatogenic activity.

This experiment was repeated with slight modification. The essential difference concerned the use of chicken pituitaries as source of gonadotropins. Beginning at 12 weeks of age, Enheptin was administered orally at a dietary level of 0.05%. Two weeks later the drug concentration was raised to 0.07% so as to assure complete inhibition of all treated individuals. The non-medicated group was limited in their feed intake to the same level as the averaged semi-weekly intake level of the other 4 groups. A non-experimental group fed *ad libitum* was used as a guide to deter-

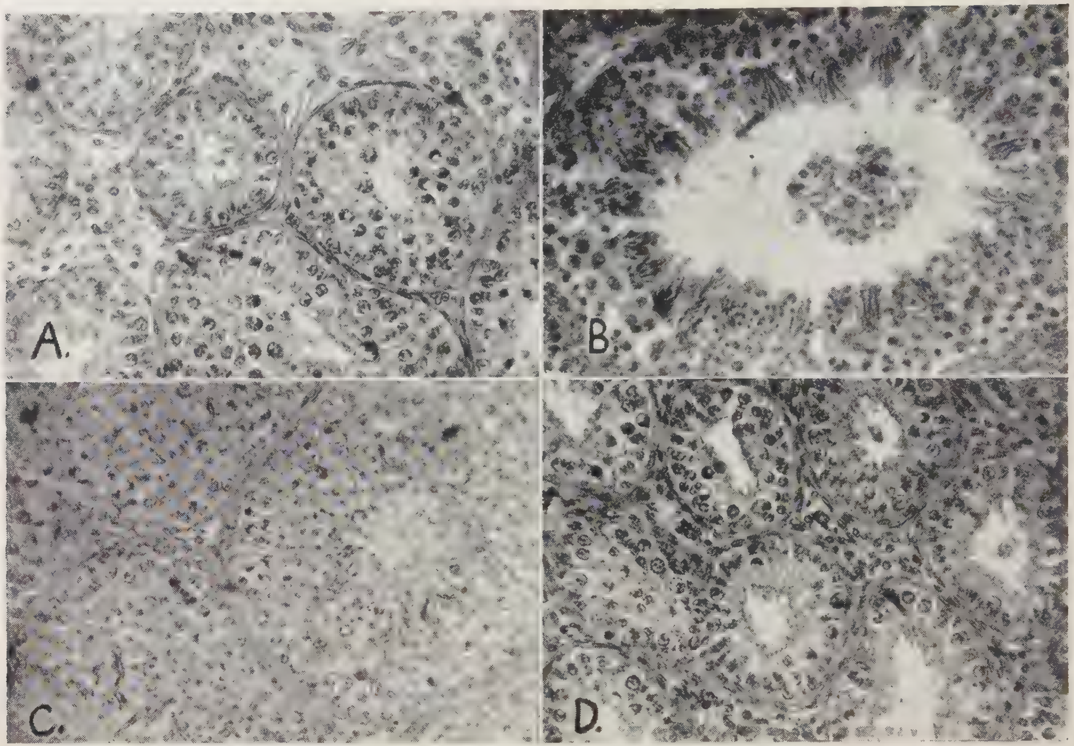

FIG. 4. Testis sections from Enheptin fed hormone treated cockerels. A) Untreated cockerel killed at end of 15th wk. Note normal but incomplete spermatogenesis. B) Untreated cockerel at 17 wk of age. Complete spermatogenesis is present. C) 15-wk-old male receiving Enheptin plus 100 u/Gonadin. Note larger tubules than shown in 4A. Note spermatid formation. D) Testis section from Enheptin treated cockerel 2 wk after hormone therapy was discontinued. There is no tubular activity as shown by diminished size and single layer of spermatogonial cells lying next to basement membrane.

mine deviations from normal growth and food consumption patterns.

Injections of Gonadin[‡] were begun simultaneously on the day Enheptin was started. Group 4 received in addition to Enheptin daily injections of a suspension of chicken pituitary powder. Each bird received the equivalent of one whole pituitary per day. This dosage was doubled during the 3rd and 4th week of treatment. Donor pituitaries were obtained from 15-month-old White Leghorn hens which had a daily flock production of about 55%. After removal, pituitaries were placed in acetone, dried as needed, powdered and suspended in distilled water to give one pituitary per cc. Each bird in Group 5 received 2.5 mg of testosterone propionate[§] daily. Injections in all groups were made alternately in either breast. At the termination of treatment, all groups were sacrificed. Organ weights taken included the comb, testes, thyroids, pituitaries and adrenals. A condensation of results is presented in Table II.

Evidence that reproductive failure due to oral administration of Enheptin could be alleviated by injection of gonadotropins was obtained when a hen which had not laid a single egg while receiving a continuous low level of the drug in the diet for 12 months after the normal onset of egg production, responded to injections of Gonadin. While getting drug treatment, she was injected with 50 units of Gonadin every other day for one week, after which time the dosage was doubled. Almost immediately her secondary sexual features developed. After 4 weeks of treatment she laid

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[‡] Gonadin is a commercial Pregnant Mare Serum produced by Cutter Laboratories.

[§] Testosterone propionate was Oreton, commercial product of Schering Corp., Bloomfield, N. J.

TABLE II. Effect of Substitution Therapy in Alleviating Sexual Suppression Due to Enheptin Feeding, 5 Groups.*

Treatment, 13-16 wk, inc.	Total % gain in body wt	Testes size,‡ mg		Avg comb wt, g
Non-medicated controlled feed intake†	30	15400	20500	41.1
		9080	10700	
E	26	613.5	715.8	10.8
		393.0	420.2	
E + 50 IU G	44	2418.6	4800	38.7
		1475.6	980	
E + CP	25	2479.2	9600.0	25.8
		1571.1	411.4	
E + 2.5 mg T	22	505.5	728.8	54.2
		295.9	409.0	

* Each group comprised 6 males.

† Feed intake was limited to a semi-weekly avg intake of the other 4 groups. E—Enheptin; G—Gonadin daily; CP—chicken pituitary, 1 whole pit. daily 13th and 14th wk, 2 pits. daily 15th and 16th wk; T—testosterone propionate daily.

‡ Numerator on left indicates actual wt avg.
Denominator " " mg/kg body wt.
Numerator on right " largest testes pair of group.
Denominator " " smallest testes pair of group.

3 eggs weighing 40, 42, and 46 g. There was one day between the first and second egg and 4 days between the second and third. She laid no eggs thereafter although hormone injections were continued for an additional 3 weeks. Five other birds of the same group receiving no hormone showed no changes during the substitution therapy applied to the single individual.

Tests for possible changes in thyroid function. Since there is considerable evidence that thyroid insufficiency will interfere with reproductive activity, 2 experiments were carried out to determine whether Enheptin interfered with thyroid secretion either directly or indirectly. Hypertrophy of the thyroid following administration of a goiterogenic agent, thiouracil, is attributed to blocking of thyroxin release and consequent hyperstimulation by the increased TSH output of the pituitary. Suppression of thyrotropin secretion by a foreign substance may then be measured by the capacity of the foreign substance to prevent thyroid hypertrophy following its simultaneous administration with goiterogenic substances. Forty-four cockerels, 9 weeks of age and in good health, were randomly divided into 4 groups. The control group received an all-mash grower diet designated N. J. No. 422. To this formula was added 0.10% thiouracil for Group 2. Group 3 received 0.10% thiouracil in the same diet plus 0.10% Enheptin, and Group 4 received 0.10% Enheptin in the diet. Body weights, comb size and feed consumption were measured weekly. At the end of 3 weeks of treatment, all birds were sacrificed and fresh organ weights recorded. Thyroids were fixed in Zenkers fluid, routinely sectioned, and stained with Hematoxylin Eosin. At autopsy all animals appeared in good health, none showing internal evidence of disease. Organ weights appear in Table III. A statistical analysis of thyroid weights revealed no significant difference in the weight of thyroids from thiouracil-treated (Group 2) birds and thiouracil-plus-Enheptin-fed (Group 3) birds. Each of these groups were significantly larger than control thyroids while those of the Enheptin-fed group (Group 4) were not different from the controls.

A further test was conducted to determine whether thyroid therapy could overcome the effects of Enheptin. White Leghorn cockerels, 15 weeks of age, were injected with thyroxin thrice weekly at the rate of 40 μg d,l thyroxin per injection for 3 weeks. They received, simultaneously, Enheptin in the diet at a concentration of 0.05%. No restoration of sexual activity accompanied thyroxin injection.

Assay for gonadotropic potency of pituitaries from Enheptin treated cockerels. Sixty-four White Leghorn cockerels hatched in May from a mass-mated flock were randomly divided into 4 groups of 14 each and treated from 9th to 12th week as follows: Group 1, intact, control diet; Group 2, intact, 0.10% Enheptin in diet; Group 3, castrate, control diet; Group 4, castrate, 0.10% Enheptin in diet. All birds were reared to 9 weeks on basal diet designated N. J. No. 422. Groups 3 and 4 were castrated (caponized) at 6 weeks of age. Feed consumption, body growth and comb area were measured weekly. All birds were sacrificed at termination of the 3-week feeding period. Each castrate bird was ex-

TABLE III. Test for Possible Thyrotropin Inhibiting Activity of 2-Amino, 5-Nitrothiazole, 4 Groups.* Body weight and comb changes represent gain or loss during treatment period. Organ weights represent final weights per kilo of final body weight.

			Organ wt in mg/kg body wt			
Treatment	% change in body wt	% change in comb area	Thyroid	Testis	Adrenal	Pituitary
C†	30.52	56.6	100.59	2243.90	137.57	7.56
T	33.52	40.0	251.55	922.73	124.83	6.85
T and E	15.10	–25.7	241.03	278.30	140.07	6.88
E	15.87	–18.0	96.00	216.91	139.59	5.17

* Each group comprised 11 cockerels.

† C—control; T—thiouracil; E—Enheptin.

amined thoroughly for residual testicular tissue. Four birds in Group 3 were confirmed as slips and were discarded. All birds appeared normal and in good health at autopsy. Assay of pituitaries was accomplished after the method of Breneman(17) which was first described by Byerly and Burrows(18). A summary of results appears in Table IV. A complete replication of this experiment with minor variations is shown in Table V. Confirmation of the results shown in Table IV is quite apparent. There is a somewhat more evident effect of pituitary suppression by Enheptin in the intact group as well as in the capons. Gonadotropin concentration per unit of tissue was significantly lower in treated than in non-treated capons. Whether or not the gonadotropic hormone content of the pituitaries of capons treated with Enheptin could be reduced to the same low level of intact Enheptin-fed males is not known.

Discussion. In several hundred White Leghorn chickens of varying ages from 3 weeks to 2½ years, including those in the present report, the oral administration of rather small quantities of Enheptin, has resulted in complete sexual involution or inhibition without exception. Following the original observation that sexual disfunction rapidly followed drug administration, an attempt was made to determine the method by which this reaction occurred. It has been shown that discontinuance of the drug was later followed by recovery of sexual activity. It must be inferred then that sexual inhibition was not permanent and therefore not due to the destruction of organs or tissues which, in any way are concerned with reproduction.

It was shown that exogenously administered gonadotropins alleviate sexual inhibition and restore sexual activity. This action was accomplished even though the pituitary was under the suppressive influence of Enheptin thus in effect simulating substitution therapy in hypophysectomy. When the exogenous hormone was discontinued, a rapid return to the sexually suppressed condition ensued. This situation has a parallel with that reported by Cranston(5) in which exogenously administered gonadotropins induced estrus in Lithospermum-treated mice. In addition it was found that not only is pituitary size reduced

TABLE IV. Assay of Pituitaries from Enheptin Treated Cockerels for Gonadotropic Potency, 4 Groups.* Each recipient chick received 5 mg equivalent of fresh pituitary tissue. Chick Unit (c.u.) represents a 35% increase over control gonad weight.

	Donors					Recipients		
Group	Body wt gain 6-11 wk, %	Change in comb area 8-11 wk, %	Final comb wt, g	Testis wt, mg/kg body wt (mg)	Pituitary size, mg	Testis wt, mg	c.u./5 mg pit.	c.u./pit.
Intact C†	106.0	55.66	28.84	5800	7.9	20.5	6.29	9.93
Intact E	92.9	–33.41	9.16	302	6.0	10.9	2.00	2.40
Capon C	105.5	–16.67	3.70	—	14.5	14.4	3.57	10.35
Capon E	80.0	–28.06	2.94	—	7.9	17.2	4.82	7.63
					Water inj.	6.4	—	—

* Each donor group consisted of 14 cockerels except Group 3 which had 10. Each recipient group comprised of 10 male chicks.

† C—control diet; E—Enheptin.

TABLE V. Replication of Pituitary Assay of Enheptin Treated Cockerels, 4 Groups.* Each recipient chick received 5 mg equivalent of fresh pituitary tissue.

	Donors				Recipients		
Group	Body wt gain 9-12 wk, %	Final comb wt, g	Testes wt, mg/kg body wt (mg)	Pituitary size, mg	Testis wt, mg	c.u./5 mg pit.	c.u./pit.
Intact C†	43.0	17.1	2153.8	8.5	18.8	3.46	5.85
Intact E	23.1	4.7	303.5	6.9	9.9	.47	.65
Capon C	43.2	1.4	—	13.7	24.6	5.41	14.82
Capon E	25.8	1.0	—	8.1	21.1	4.23	6.86
				Water inj.	8.5	—	—

* Each donor group consisted of 20 cockerels. Each recipient group consisted of 10 male chicks.

† C—control diet; E—Enheptin.

significantly in Enheptin-treated chickens, but so is the total pituitary gonadotropic hormone potency.

Somewhat related to the foregoing is the problem of species specificity. Three attempts have been made (Pino, unpublished) to repeat our observations on sexual suppression with rats, all without success. Indeed, there is some evidence that even the turkey responds somewhat differently than the domestic chicken.

Summary. Administration of gonadotropic substances simultaneously with Enheptin (2-amino, 5-nitrothiazole) sustained sexual activity in most cases. Failure to restore sexual normality in all cases is probably due to the quality and quantity of administered gonadotropic hormone. The drug Enheptin was not able to suppress thyroidal hypertrophy during thiouracil treatment. Pituitary gonadotropic hormone potency is reduced in intact as well as capons treated with Enheptin.

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Survival of Tumor Homografts in Mice Pretreated with Ultrafiltrates of Hyaluronidase-Digested Tumor Supernatant.* (21336)

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The natural resistance of mice of a given inbred strain to homografts of mouse tumors can be abrogated by injecting extracts or homogenates of *normal or cancerous tissues* of mice into the prospective host prior to the grafting of live tumor. (For bibliography, see (1)) (*Homografts* are defined as grafts of tissues, indigenous to one inbred strain of mice, into mice of unrelated inbred strains.) A number of investigations has been reported(1-5) dealing with attempts at chemical and physical characterization of the active principles in the tissue homogenates responsible for "conditioning" the host to accept the foreign tumor graft. One type of analysis has used the differential ultrafiltration of various tumor preparations and extracts as a criterion for ascertaining the physical state of the active principles. Thus the so-called "XYZ factor" in fresh tumor has been reported to be unfiltrable through Berkefeld or Seitz filters unless the tumor first is stored in the frozen state for long periods of time(2); the "enhancing factor" in a lyophilized tumor preparation has been reported to be filtrable through Seitz filters but not Berkefeld or Selas filters(3); the mouse mammary cancer "fresh accelerant" has been reported to be unfiltrable through a Seitz filter(4); and the "conditioning substances" from a mouse tumor extract have been reported to be filtrable through Pyrex glass ultrafine fritted discs, but not through Selas or Berkefeld filters(5).

We have emphasized previously(5) that such reported differences are not necessarily attributable to differences in the active principles themselves, but may be due to the complexes in which they must naturally occur. In this light, it appeared to us that the reported differences in results obtained both in this laboratory and elsewhere(1-5) might be ascribed to variations in the techniques used for preparing the tissue extracts prior to filtration. Since Bryant, Earle, and Peppers(6) had obtained easily filtrable chicken embryo extracts by first treating them with hyaluronidase, and since our filtration problems seemed to parallel theirs, we investigated hyaluronidase treatment of tumor extract as a possible remedy for our previously reported difficulties (5) in getting the supernatants to pass through microporous filters.

Materials and methods. The tissue used as the source of the active principle ("conditioning substance") and for homografting, was the transplantable tumor Sarcoma I, which is indigenous to the inbred A strain of mice. Hosts were from the C57BL/6Ks strain, in which grafts of Sarcoma I rarely survive in untreated mice. The animals were 2-4 months old at the start of the experiments. Prior to tumor grafting, each mouse received a series of 4 intraperitoneal injections (0.5 ml per injection), over a 2-week period, of the various preparations of Sarcoma I extract (to be described below). One week after the last injection, the animals received, by trocar, bits of live tumor which were implanted subcutaneously in the interscapular region, under aseptic conditions. The course of growth of the grafts was followed by periodic palpation until the animals either died with a progressively growing tumor or showed no gross signs of a graft for a consecutive period of 2 months, at which time they were sacrificed as negative. The *tumor extracts* were prepared in 2 concentrations by high-speed centrifugation, according to a method previously described(5). *Full-strength* (F.S.) supernatant was derived from a homogenate of approximately equal weights

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of freshly secured tumor and 0.85% NaCl, which was centrifuged for 20 min. at an average RCF of 8500 x g. *Dilute* (D.S.) supernatant was prepared from the F. S. supernatant, which was diluted 1:10 with 0.85% NaCl and then centrifuged for 30 min. at an average RCF of 11000 x g. Further treatment of the extracts is described in detail below for each of the experiments. In *Exp.* 1, hyaluronidase‡ was combined with D.S. supernatant (0.1 mg/ml) and the mixture maintained at room temperature for 4 to 5 hours. This was then centrifuged at an average RCF of 8000 x g for 15 min. A portion of the supernatant thus obtained was then passed through an ultrafine fritted glass (Pyrex) disc (maximum pore diameter 1.2μ). None of the difficulties with clogging of the filters which we had previously encountered with untreated tumor supernatant(5) was experienced here, and the material passed through the filter at a rate approaching that for water. The remaining portion of the supernatant was used unfiltered as control material. Both portions were used for injection without further treatment. For *Exp.* 2, hyaluronidase§ was combined with F.S. supernatant (1 mg/ml). The mixture was maintained for 4-5 hours at room temperature and then centrifuged at an average RCF of 11000 x g for 20 min. A portion of the supernatant was filtered through a #03 Selas porcelain tube (maximum pore diameter 1.2μ) and another portion through a Seitz S-1 asbestos filter. A third unfiltered portion was used as control material. Filtration was rapid through the Selas filter, and somewhat slower through the Seitz filter. All 3 portions were then diluted 1:10 with sterile 0.85% NaCl for injection. In *Exp.* 3, D.S. supernatant was combined with hyaluronidase§ (0.1 mg/ml) and kept at room temperature for 4-5 hours. The digest was then centrifuged at an average RCF of 8000 x g for 15 min. Portions of the supernatant were filtered either through a Berkefeld N filter (calibrated as passing 43 ml of distilled water in 5 min. at a pressure of 25.9 cm of mercury), a Seitz S-1 asbestos filter pad, or a #03 Selas porcelain filter. The material passed through the Selas filter at a rate approaching that for water, and less rapidly through the Berkefeld filter. There was a marked retardation in the Seitz filter. All the preparations were used for injection without further treatment. For all 3 experiments, the preparations were stored in the deep-freeze (−26°C) and thawed as needed. The nitrogen content of each filtered and control preparation was estimated by a Pregl modification(7) of the micro-Kjeldahl procedure. Determinations were made in duplicate with 1.00-ml portions of each of the preparations used for injection.

Results. (Table I). Hyaluronidase was found to have no effect upon the activity of the conditioning substances responsible for abrogating the normal resistance of the host to the tumor homografts. On the other hand, the enzyme was found to change the tumor supernatant to a state much more easily filtrable through various microporous filters. Even with the slower filtration encountered with the Berkefeld N and Seitz S-1 filters (in which more than half of the nitrogen-containing material was held back) a slightly active filtrate was obtained. This was evidenced by the more marked growth of the grafts before regression in mice treated with these materials, as compared with the uninjected controls or the animals that received only hyaluronidase.

In *Exp.* 1, the nitrogen content of the hyaluronidase digest of the D.S. supernatant before centrifugation was 0.574 mg N/ml, and after centrifugation was 0.517 mg N/ml. Thus, approximately 10% of the nitrogen-containing material in the D.S. supernatant was rendered sedimentable, presumably as a result of a decrease in viscosity of the supernatant following hyaluronidase treatment. In all 3 experiments, the *filtrates* exhibited a further loss of nitrogen-containing material (Table I), the degree of loss corresponding to the observed differences in filtration rate for each of the different types of filters. The relative order of retention of the filters (in terms of increasing amounts of nitrogenous materials passed) was: Seitz, Berkefeld N, Selas, and fritted glass disc.

In *Exp.* 2, despite a relatively high nitrogen

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‡ Nutritional Biochemical Corp., Cleveland, O.; assayed at 100-200 TRU/mg.

§ Worthington Biochemical Corp., Freehold, N. J., assayed at 150-250 TRU/mg.

TABLE I. Growth of Homografts of Strain A Tumor, Sarcoma I, in C57BL/6Ks Mice Receiving Prior Injections of Sarcoma I Supernatants that Had Been Digested with Hyaluronidase and then Passed through Micro-porous Filters.

Material injected, treatment, amount administered Material injected*	Filter used	Total mg N inj./mouse	Hosts dying with tumors†
Exp. 1			
Tumor supernatant‡	Ult‖ glass disc	.88	9/10
” ”	Unfiltered	1.03	10/10
Hyaluronidase	Ult glass disc	.02	0/5
None	—	—	0/5
Exp. 2			
Tumor supernatant§	Seitz S-1 asbestos pad	.64	1/5
Idem	Selas #03 porcelain	.84	2/5
”	Unfiltered	1.56	3/5
None	—	—	0/5
Exp. 3			
Tumor supernatant‡	Seitz S-1 asbestos pad	.29	0/4
Idem	Selas #03 porcelain	.62	5/5
”	Berkefeld N	.36	0/5
”	Unfiltered	.79	4/5
None	—	—	0/5

* Administered intraperitoneally in 4 inj. of 0.5 ml each, given twice weekly. Live tumor grafted 1 wk after last inj.

† Numerators are No. dying; denominators, total No. of mice in each group.

‡ Tumor supernatant prepared in a 1:10 dilution with 0.85% NaCl from "full strength" supernatant *before* digestion with hyaluronidase.

§ "Full strength" tumor supernatant digested with hyaluronidase; diluted 1:10 with 0.85% NaCl *after* digestion.

‖ Ult = Ultrafine fritted.

content, the unfiltered and filtered extracts showed a lower activity than comparable materials used in *Exp.* 1 and 3. This difference is most certainly due to variation in the procedures used for preparing the extracts. (In *Exp.* 2, *F. S. supernatant* was treated with the enzyme, while in *Exp.* 1 and 3, the *D.S. supernatant* was digested).

Discussion. Digestion of the tumor supernatant with hyaluronidase effected a marked improvement in the filtrability of the "conditioning substance" through the fritted glass and Selas porcelain filters, as compared with our previous experience(5). Treatment with the enzyme prior to centrifugation apparently facilitated the removal of particulate material, and although some of the "conditioning substance" was undoubtedly coprecipitated with the particulate matter, enough remained in the supernatant to give potent, biologically active preparations. Nevertheless, the slower rates of filtration through the Seitz and Berkefeld N filters, together with a marked loss of activity in the latter filtrates, indicates that the active agent was not completely dissociated by hyaluronidase from the complex in which it originally is bound.

It has been shown that the "conditioning substance" has a high affinity for particulate matter, heat-denatured proteins, and for proteins adsorbed by Lloyd's reagent(5). The degree to which these potential adsorbents are present in different tissue preparations would therefore be a decisive factor in the amount of active substance recovered in ultrafiltrates. It would thus appear that the use of ultrafiltration as a criterion for establishing possible similarities or differences among the so-called XYZ, enhancing, fresh accelerant, and conditioning substances, is of little value where tissue homogenates or crude extracts (even those treated with hyaluronidase) are the materials that are being filtered.

Summary. Tumor extracts obtained by centrifugation, if incubated with hyaluronidase and recentrifuged, will pass readily through Pyrex glass ultrafine fritted discs and Selas #03 porcelain filters. They pass more slowly through a Berkefeld N filter, and least rapidly through a Seitz S-1 asbestos filter, with over

50% loss of nitrogen-containing material. Filtrates from the first two filters, when injected into the prospective host prior to implanting a tumor homograft, are fully effective in abrogating the animal's normal resistance to the graft. Filtrates of the latter 2 filters show a slight effect, in terms of greater growth of the homografts prior to regression, as compared with uninjected controls.

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Toxoplasmosis in Swine and Rodents. Reciprocal Oral Infection and Potential Human Hazard.*† (21337)

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The epidemiology of toxoplasmosis is not yet clearly understood although the infection is widespread in man and animals. In the United States 20 to 40% or more of the adult population have given serological evidence of past or present infection with *Toxoplasma*(1,2). If this evidence is reliable, then human infection is widespread here, even though human disease is not common because most persons tolerate the infection well.‡ Current thought assumes that the infection is conveyed in some undefined manner from animals to man perhaps by the intermediary of arthropoda, or by droplet infection, or the handling of infected animals, or through soiling of food by excreta, particularly of dogs, or, finally, by ingestion of contaminated food.

This article is the first report of a long-term investigation designed to test the hypothesis that toxoplasmosis may be contracted by eating pork, and that swine in turn can become infected by feeding on infected tissue, the source of this tissue being either other swine or rodents, in particular, rats. Essentially, the concept is that feeding cycles may be involved in toxoplasmosis which are similar to those known for trichina. The likelihood of such a cycle is suggested by previously known facts: 1. So far as known, toxoplasma infects all warm blooded animals and spontaneous infection has been reported in a variety of domestic animals; 2. Toxoplasma infection is transmissible *per os* to mice as first reported in 1913(3) and since repeatedly confirmed. 3. Rats trapped in American cities have frequently been found to be spontaneously infected, with an incidence as high as 8%(4). 4. Animals remain infectious for long periods after the initial acute toxoplasmosis has terminated(5). It seemed to the present authors that these facts integrated themselves in a suggestive fashion in the light of above-cited hypothesis and that the latter warranted in-

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‡ Positive serological tests for *Toxoplasma* have been reported in *Sarcocystis* infections. *Sarcocystis* is so rare in man however, that the evidence for widespread *Toxoplasma* infection is still valid.

TABLE I. Response of Pigs to Feeding of Toxoplasma (Porcine Strain).

Dye test titer before feeding*	Day fed†	Infected material fed	Dye test after feeding Day	Titer	Infections proved by isolation of organism	Tissues found infected	Min duration of infection (days)	Comment
0	1	½ rat brain	36	1	no	—	—	Infection not proved
	62	mice (2)	69	64				
	119	mouse organs	160	0				
1	1	½ rat brain	36	1	no	—	—	*Idem*
	62	mouse organs	69	1				
			93	64				
0	1	mouse organs	17	4096	yes	brain	27	
	3	" "	42	4096				
	4	" "						
	8	2 mice						
	11	2 "						
	15	1 mouse						
0	Between 1 & 53 fed 12 times	mice or mouse tissue	17	4096	yes	brain	19	
			46	1024				
			55	256				
1	1	rat brain	45	1	no	—	—	Dye test equivocal; animal skin tested on 36th day
	2	*Idem*	57	256				
	(twice)	"	63	64				
	5							
0	1	"	30	256	no	—	—	
			44	256				
			69	256				
			118	64				
0	1	pig lung	7	0	yes	blood on day 7	alive	
	2	*Idem*	26	4096				
			82	64				
			158	64				

* Reciprocal of titer.
† Day 1 is the day of first feeding.
All animals in this table were between about 15 and 35 lb when first used.

vestigation. While this work was in its initial stages, an epidemic of toxoplasmosis in swine was reported from Ohio(6).

Materials and methods. Two strains of *Toxoplasma* were employed; one of porcine origin served in all infection experiments, whereas in the dye test the RH strain was used. The dye-test technic employed was that of Feldman(1); serum was prepared in 4-fold serial dilutions and titers given as highest dilution inhibiting staining of more than half of the toxoplasmata. Pigs raised and purchased in the New Haven area were fed antibiotic-free stock food. Most of these animals were utilized shortly after weaning, all before 9 months of age and, for convenience, were usually kept less than 6 months. All feedings of swine and rodents were voluntary, no gavage nor stomach tube was employed.

Results with swine. Pigs became infected after eating infected tissue from other swine or from rodents (rats and mice) (Table I). In some swine, infection was proved by isolation of toxoplasmata. In others, infection was strongly suggested by a rise in dye-test titer from negative, or positive in undiluted serum only, to a post-feeding level of 1 in 256 to 1 in 4096.

That these levels are to be considered significant, under the condition of the experiments, is concluded from the data in Table II which show that almost all pigs inoculated with Toxoplasma develop dye-test titers of 1 in 256 or higher.

One "false negative" dye test was encountered; despite this, the dye test detects the greatest number of swine presumed to be infected due to prior inoculation of toxoplasmata. Subinoculation of pig tissue into mice, a method of choice for proving infections, de-

TABLE II. Response of Pigs to Injection of Toxoplasma (Porcine Strain).

Dye test titer before inoculation†	Inoculation			Clinical symptoms	Dye test after inj.		Infection proved by isolation of organism	Tissues found infected	Min duration of infection (days)	Comment
	Day	Route	Dose		Day*	Titer†				
(a) Young pigs, none over about 35 lb										
1	1	i-abd.	2.4×10^7	—	6	1	yes	brain	34	
					12	1				
	64	”	3.1×10^7		21	neg.				
					71	64				
					98	256				
0	1	”	2.4×10^7	—	6	1	”	blood on day 6	41	Infection proved but no rise in dye-test titer
					12	1				
					21	1		lung on day 42		
					41	1				
256	1	”	1.59×10^7	—	11	1024	not tested	not tested	not tested	High pre-inoculation dye-test titer
64	1	”	1.59×10^7	—	11	1024	”	”	”	Pre-inoculation dye-test titer elevated
0	1	i-card.	(1)	Marked; died on day 6	—	—	Not tested; death from generalized toxoplasmosis	Lung, heart, brain, liver, spleen, striated muscles (ham)	—	Youngest animal and only fatal infection; weighed approx. 15 lb when inj.
(b) Older animals										
1	1	i-card.	3.8×10^8	Temporary	15	256	not tested	not tested	—	Animal approx. 7 mo older when inoculated than others in series; sacrificed 2 wk after inoculation
1	1	”	8×10^7	—	8	1024	no	no	—	Approx. 4 mo older when inoculated than others
					88	1024				
	78	”	4×10^7		105	1024				

(1) Not counted. Probably between 1×10^7 and 1×10^8.

* ‘‘Day 1’’ is the day of first inoculation, subsequent intervals are counted from this day.

† Reciprocal of titer.

tected the next largest number. The skin test antigen as prepared for human use has not been serviceable, giving no clearcut results.

Tissue sections have not revealed organisms except in one fatal infection. In general all pigs have tolerated toxoplasma infection very well following either feeding or intra-abdominal injection of as many as 3 x 10^7 organisms in younger animals and up to 3.8 x 10^8 toxoplasmata in older pigs. The one fatality followed intracardiac inoculation of a very young animal of approximately 15 lb weight. Death occurred in 6 days and postmortem findings showed acute generalized form of the disease.

Of possible high significance is the finding that the healthy-appearing swine continued to harbor virulent organisms in brain and lung tissue for at least 41 days, thus far the longest interval after which we have made tests. The maximum survival period of *Toxoplasma* in swine is very probably much greater, perhaps as long as several years, to judge from the persistence of the organism in tissues of other animals(2). That such persistence would be an advantage in a transmission cycle seems likely.

That spontaneous infection occurs locally was suggested by findings in 2 pigs. These gave pre-inoculation dye-test titers of 1 in 64 and 1 in 256 (Table II). These pigs were raised in the vicinity of New Haven, and proved to have been fed uncooked garbage. In addition, their pens were overrun with rats, even in daytime, and trapped in large numbers daily.

TABLE III. Response of Rats to Intra-abdominal Inoculation of Toxoplasma (Porcine Strain).

Day*	Post-inoc. dye test Day	Post-inoc. dye test Titer	Organisms isolated post-mortem	Tissue infected	Min duration of infection (days)
1 3 7	97	256	yes	brain	98
1, 7, 10	199	256	”	”	189
Idem	200	64	”	”	190
”	204	256	”	”	194
1, 7, 11	266	256	n.t.†	—	—
Idem	211	1024	yes	brain	200
1, 3, 7	106	256	no	—	—

* Day 1 is day of first injection.
† n.t. = not tested.
These rats weighed 100 g each.

Toxoplasmosis of Albino Rat. Following feeding of rodent and pig tissues. Eyles reported that the dye test for toxoplasma "is not a reliable indicator of the existence of organisms which can be recovered from the rat" (7), because he found negative titers coexisting with proved toxoplasma infection. While the dye test was being evaluated for our purposes, one group of rats was put under experiment. These animals were fed infected mouse tissue and subsequently tested for infection only by subinoculation of brain tissue into mice. Even by this procedure it was possible to prove that approximately 20% of 21 rats became infected. The true incidence may be higher than these figures indicate, since it is possible to test only a small portion of brain in the manner described. Meanwhile a series of dye-test titers obtained from inoculated animals gave levels of 1 in 64 or more. These titers were found 3 to 6 months following injection, and the re-

TABLE IV. Response of Rats to Feeding of Toxoplasma (Porcine Strain).

Wt, g	Day fed*	Infected material fed	Dye test after feeding Day	Dye test after feeding Titer	Infection proved by isolation of organism	Tissues found infected	Min duration of infection (days)
170	1, 2	ham	70	64	n.t.†	—	—
”	”	”	70, 158	256	yes	brain	158
”	”	”	70	neg.	n.t.	—	—
”	”	pig lung	”	”	n.t.	—	—
”	1	” heart	”	”	n.t.	—	—
”	1, 2	” liver	”	”	n.t.	—	—
80	1, 2, 5, 13	rat brain	42	”	no	no	—
254	1, 14	” ”	52	”	n.t.	—	—

* Day 1 is the day of the first feeding.
† n.t. = not tested.

sults indicated that the test would be useful for our work (Table III).§

All rats subsequently used were dye-tested after feeding. It will be seen from Table IV that although we have only 8 animals, 2 of these, fed infected ham, have given dye-test titers to a level considered significant when tested 10 weeks later. Thus, of a total of 29 rats, 6 or 20% have given evidence, or proof, of infection after feeding on swine or rodent tissue.

With rats, as with swine, the oral route proved relatively inefficient when compared with inoculation procedures. This, of course, was to be anticipated(2). Some rats became infected after single feedings, but many did not, and, at roughly the same dose at each feeding, the number of animals infected increased with repeated feedings. There is reason to believe that other strains of toxoplasma may prove more infective when fed (8) and this will be tested. In any case, repeated exposure will increase the number of infections, and such repetition seems favored by the chronic type of toxoplasmosis which develops in rats, some of which will eventually serve as food to other rats and swine. (Tables III and IV).

The experimental results thus far achieved favor the hypothesis which initiated the present investigation: that toxoplasmosis may be contracted by eating pork, and that swine can become infected by feeding on infected tissue, the source of this tissue being either other swine, or rodents.

Discussion. Whether man is susceptible to infection by the oral route can hardly be put to test, but the matter will be investigated in primates. However, in so far as humans are concerned, there is little doubt that under present circumstances they are offered the opportunity to infect themselves through the sale of infected swine. If we realize that the American public consumes sufficient undercooked pork to infect more than one person in six and perhaps as many as one in three with trichina(9,10), it appears that the toxoplasma hazard merits investigation. That the risk from infected pork is of the same magnitude with toxoplasma as with trichina is not to be inferred from the foregoing; notably toxoplasma does not survive for longer than 30 days under refrigeration, so far as known(11,12).‖ The true risk will depend upon survival periods in refrigerated pork cadavers, the interval between slaughter and consumption, initial infection rates in nature, and other factors. These problems will be investigated in which emphasis will shift from the experimental study of swine infections to an attempt to evaluate the extent to which pork can be implicated as a source of possibly widespread *Toxoplasma* infection.

Human infection may be determined principally by such a feeding cycle or one similar to it. In the epidemiology of animal toxoplasmosis, additional explanations are required. Man, other omnivores and carnivores may infect themselves by feeding on tissue which contains toxoplasma, but it seems unlikely that a mechanism of a similar sort can produce the widespread infection and occasional epidemics noted among herbivores and grain eating animals.

Summary. The customary method of transmission of toxoplasmsis to man and to animals remains unknown. This report shows that infections are transmitted between swine and rodents when either animal is fed infected tissue from the other, likewise when pigs ingest infected swine offal. The feeding cycles described are reminiscent of those known in trichinosis. Similarities and differences are discussed, and implications for human disease pointed out. Whether there is a risk to the public, and if so, its extent, will require future studies on swine and on pork, under natural as well as experimental conditions.

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§ Eyles, who used trapped rats, was perhaps dealing with much older infections. In his animals, titers may have dropped to low or undetectable levels.

‖ Recently we have obtained survival for at least 60 days at −70°C.

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Sterol Specificity of Pancreatic Cholesterol Esterase.* (21338)

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Recent papers(1-4) from these laboratories have reported procedures for investigating the esterifying and hydrolyzing cholesterol esterase systems and the occurrence and characteristics of both systems in pancreatin, dog serum and rat intestinal mucosa. It was also observed(5) that soybean sterols were esterified *in vitro* under the same conditions as cholesterol and that they inhibited dietary-induced hypercholesterolemia in rats. The assumption was made that soybean sterols lower blood cholesterol by competing with cholesterol for cholesterol esterase, fatty acids and bile salts. In view of these findings, and the possible role of cholesterol esterase in the absorption of cholesterol(6), it seemed desirable to determine the sterol specificity of both the esterifying and hydrolytic cholesterol esterase systems of pancreas.

Methods and materials. Substrate mixtures were prepared as described in an earlier report (2). The substrate for esterification was 100 mg sterol and 3 equivalents of fatty acid, while that for hydrolysis was an amount of ester equivalent to 100 mg of free sterol. The pH of the digests for hydrolysis was 6.6; for esterification with butyric acid, pH 5.2; for esterification with oleic acid, pH 6.1. One ml of hog pancreas homogenate equivalent to .1 g fresh tissue was added to 12 ml of substrate mixture in all cases. The incubation temperature was 37°C. Samples were removed from the enzyme digests (substrate mixture plus pancreas homogenate) at 0, 2, 4, and 24 hours. Free and total unsaturated sterols were determined in the same manner as cholesterol, *i.e.*, digitonin precipitation followed by the Lieberman-Burchard color reaction(7) and comparison with standard solutions of sterols. Dihydrocholesterol† was determined by precipitation with digitonin followed by weighing of the digitonide. All calculations were based on the amount of free sterol present at zero time and at the end of the incubation periods. The butyrate esters were synthesized by refluxing the various sterols with butyric anhydride for 2 hours at 150°C. The esters were recrystallized from absolute alcohol 5 times. The clear melting points (uncorrected) were as follows: cholesterol butyrate, 114°C; β-sitosterol butyrate, 93°C; sitosterol butyrate, 95°C; stigmasterol butyrate, 113°C; ergosterol butyrate, 121°C; and dihydrocholesterol butyrate, 85°C. The sterol oleates were prepared according to the method of Page and Rudy(8). The clear melting points (uncorrected) were as follows: cholesterol oleate, 44°C; sitosterol oleate, 39°C; stigmasterol oleate, 41°C; and ergosterol, 42°C. The esters gave no precipitate with digitonin and were stored in a vacuum desiccator over concentrated sulfuric acid.

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† Generously supplied by the Schering Corp., Bloomfield, N. J.

TABLE I. Enzymatic Esterification and Hydrolysis of Sterols and Sterol Esters by Pancreatic Cholesterol Esterase.

	Esterification				Hydrolysis		
Substrate*	2 hr	4 hr	24 hr	Substrate*	2 hr	4 hr	24 hr
		%				%	
†di-oleic a.	85.5		85.7				
di-butyric a.	44.2		69.9	di butyrate	77.4		81.2
ch-oleic a.	57.7	72.4	80.2	ch oleate	30.7	34.0	46.5
ch-butyric a.	.0	.0	33.3	ch butyrate	92.6	95.0	95.0
β-si-oleic a.	23.7	39.6	74.5				
β-si-butyric a.	.0	.0	.0	β-si butyrate	92.0	99.0	99.0
‡si-oleic a.	22.1	41.6	67.0	si oleate	28.3	31.9	57.6
si-butyric a.	.0	.0	.0	si butyrate	83.6	98.9	98.9
st-oleic a.	8.7	12.5	25.6	st oleate	23.7	31.7	98.2
st-butyric a.	.0	.0	.0	st butyrate	81.2	89.3	90.8
er-oleic a.	6.9	14.7	24.7	er oleate	9.4	14.1	23.0
er-butyric a.	.0	.0	.0	er butyrate	22.8	29.0	55.8

* Substrate for esterification was 100 mg of free sterol and 3 equivalents of fatty acid, while that for hydrolysis was an amount of ester equivalent to 100 mg free sterol. Bile salts, 1 ml 14% sodium taurocholate. Enzyme, 1 ml 10% hog pancreas homogenate.

† di = dihydrocholesterol; ch = cholesterol; β-si = β-sitosterol; si = sitosterol; st = stigmasterol; er = ergosterol.

‡ Mixture of α, β, γ sitosterols.

Results. In a previous study(9) it was observed that pancreatic cholesterol esterase synthesized and hydrolyzed a large number of cholesterol esters. The activity in both systems varied with the chain length of the fatty acid. The higher fatty acids were more rapidly esterified than the short-chain acids, while the short-chain cholesterol esters were more rapidly split than the long-chain esters. Also, the optimum pH of the esterifying reaction was found to vary with the fatty acid component. In the present study a short-chain fatty acid (butyric acid and butyrate) and a long-chain acid (oleic acid and oleate) were chosen as substrates in order to encompass the wide range of substrate specificity of the enzyme. The esterification with butyric and oleic acids was carried out at the respective optimum pH for these acids, namely, 5.2 and 6.1.

The esterifying and hydrolyzing activity obtained with the different sterols and sterol esters at various times is shown in Table I. All of the sterols tested were esterified with oleic acid, while only cholesterol and dihydrocholesterol were esterified with butyric acid. The order of esterifying activity observed with oleic acid was as follows: dihydrocholesterol > cholesterol > β-sitosterol > sitosterol > stigmasterol > ergosterol.

In Table I are also shown the results obtained with the hydrolytic system. The hydrolysis of the butyrate esters was very rapid with the exception of ergosterol butyrate; 77.4 to 92.6% of the butyrate esters were split in the first 2 hours. The oleate esters were split at a considerably slower rate. The following order of hydrolytic activity was observed for the sterol oleates: cholesterol oleate > sitosterol oleate > stigmasterol oleate > ergosterol oleate. After 2 hours the rate of hydrolysis of these esters leveled off with the exception of stigmasterol oleate which was virtually all split at the end of 24 hours. The increased rate of hydrolysis of the latter ester was unusual, but was confirmed in several additional experiments. In comparing the esterification of the sterols with oleic acid and the hydrolysis of the corresponding sterol oleates, it can be seen that the reaction was markedly in favor of esterification with respect to dihydrocholesterol and cholesterol, while with the other sterols the reaction was either equal in both directions or in favor of hydrolysis. In the butyric acid-butyrate system, the reaction in all cases was in favor of hydrolysis.

Discussion. The sterols tested all possessed the same nucleus and differed only in the number of double bonds and the structure of the side chain. The activity with the different sterols in the esterifying system was markedly influenced by the number of double bonds in the ring and side chain. Dihydrocholesterol with no double bonds was more active than cholesterol which has one double bond in ring two. Similarly, β-sitosterol with no double bonds in the side chain was more rapidly esterified than stigmasterol which has one double bond in the side chain. The addition of an ethyl group in the side chain, as in β-sitosterol, diminished the activity when compared to cholesterol. These differences due to structure were not as evident in the hydrolytic system. In the present study epimers were not tested, but it is of significance that Nedswedski(10) reported that coprosterol, epi-coprosterol and epi-dihydrocholesterol were not esterified by pancreatic cholesterol esterase. The ease of formation of the enzyme-substrate-bile salt complex is probably the factor in determining the rate of esterification and hydrolysis.

Summary. Pancreatic cholesterol esterase catalyzes the esterification and hydrolysis of a number of different sterols and sterol esters. The order of activity observed in the esterifying system with oleic acid was as follows: dihydrocholesterol > cholesterol > β-sitosterol > sitosterol > stigmasterol > ergosterol. Of these sterols, only dihydrocholesterol and cholesterol were esterified with butyric acid. In the hydrolytic system all of the sterol butyrates tested were split very rapidly, with the exception of ergosterol butyrate. the order of splitting of the oleates in the first 4 hours was: cholesterol oleate > sitosterol oleate > stigmasterol oleate > ergosterol oleate. The relation of enzyme activity to sterol structure is discussed.

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Potentiating Effect of Iproniazid on Action of Some Sympathicomimetic Amines.* (21339)

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It has been shown that iproniazid (1-isonicotinyl-2-isopropyl hydrazine) blocks amine oxidase (MO) *in vitro*(1) and *in vivo*(2,3). Since MO is able to inactivate sympathicomimetic amines by oxidative deamination, the question arose whether by inhibiting MO with iproniazid, the metabolism and pharmacologic action of such amines could be influenced. A suitable animal for such studies was found in the guinea pig which produces a characteristic syndrome of intoxication. An attempt was made to affect this response by pretreatment of the animals with iproniazid. The results are presented in this paper.

Materials and methods. Female guinea pigs,

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weighing 220 to 520 g (average 400 g) in good general health, were used in these experiments. All drugs were administered intraperitoneally except epinephrine, which was given subcutaneously. Two hours before administration of the investigated amines iproniazid (150 micromoles/kg body weight) or isoniazid (350 micromoles/kg) was injected. In some experiments different doses were employed. The body temperature was measured rectally.

Results. 1. *Effect of amines on guinea pigs.* With increasing doses of the various amines used in these studies toxic manifestations were produced. Although there was some variation in the individual amine, the average response was directly proportional to the dose and was recorded in the following manner: 1+: Tachypnea, contraction of the masseters, shaking of the head in vertical direction, shivering and pilomotor stimulation. 2+: Twitching of the skeletal musculature, increased salivation, urination, squealing. 3+: Clonic, clonic-tonic fits, opisthotonus, lacrimation, exophthalmos. 4+: Tonic convulsions, loss of balance, loss of tonus, jumping, akinesis.

The following should be noted: Contraction of the masseters or opisthotonus was noted only in guinea pigs given epinephrine, ephedrine and phenylethylamine. When pretreated with iproniazid, ephedrine and occasionally tyramine induced the animals to scratch their skin during the 1+ reaction. Tyramine produced much milder reactions than the other amines. Epinephrine is biologically so active that amounts which would produce noticeable neurological side effects are lethal when administered in single injections. When a dose of 9 micromoles of epinephrine was administered in a continuous drip to an immobilized guinea pig 1+ to 3+ reactions (except fits) could be noted.

TABLE I. Effect of Isonicotinic Acid Hydrazides on Response of Guinea Pigs to Phenylethylamine (PEA).

	Without hydrazides, 20 animals per series	With 150 μmoles/kg iproniazid, 10 animals per series	With 350 μmoles isoniazid	
Neurological response	Avg of μmoles PEA		No. of animals	Avg of μmoles PEA
1+	450	30	10	120-250
2+	600	60	6	200-300
3+	750	75	10	600*

* No lacrimation.

2. *Effect of hydrazides on the response to phenylethylamine.* The response of the animal to phenylethylamine is very markedly increased by the pretreatment of iproniazid. Only one-tenth of the amine is necessary to produce response 3+ as compared with the unpretreated animals (Table I), while a higher dose of isoniazid has little or no effect.

3. *Effect of iproniazid on the response to other monoamines.* In contrast to phenylethylamine, the response to ephedrine and D-a-methylphenylethylamine was not potentiated by iproniazid. In order to produce a neurological response of 2+, 0.6 millimole/kg of ephedrine or 0.05 millimole/kg of methyl phenylethylamine were required. Using 5 guinea pigs for each group and 20 animals *in toto,* no difference in response was caused by 150 millimoles/kg of iproniazid. Most of the guinea pigs pretreated with iproniazid developed symptoms of severe itching after a dose of 49 micromoles of ephedrine per animal. Isoniazid seemed to be without effect. The administration of 5.0 to 12.0 millimoles of tyramine per kg to 10 animals produced a syndrome of the 2+ degree, while 5 millimoles killed 6 animals, when administered to iproniazid pretreated animals.

4. *Effect of application of hydrazides and amines on body temperature.* The application of ephedrine or phenylethylamine produces a depression in body temperature, while the hydrazides are without effect. This depression of body temperature by amines is converted into an increase through the pretreatment of the animals with hydrazides (Table II).

Discussion. The intensity of the toxic syndrome produced by phenylethylamine is a function of the amount of amine applied (Table I). Since this amine is a substrate of MO, its level and, therefore, its action should be affected by the activity of MO. This is indeed the case. After blocking MO entirely by iproniazid (see introduction) the pharmacological response to phenylethylamine was much more severe. Since a-methyl substituted amines are not substrates of MO(4), the ac-

TABLE II. Effect of Combined Application of Hydrazides and Amines on Body Temperature.

Amines	Hydrazides	No. of animals	Temp, °C
μmoles/kg	μmoles/kg		
300 Phenylethylamine	—	10	38.0 ± .5
294 Ephedrine	—	10	38.0 ± .2
60 Phenylethylamine + 296 Ephedrine	—	7	37.6 ± .5
—	150 Ip*	20	38.6 ± .4
—	350 Is	15	38.5 ± .5
300 Phenylethylamine	150 Ip	20	39.9 ± .9
300 "	350 Is	10	39.6 ±1.0
296 Ephedrine	150 Ip	10	38.4 ± .4
—	—	30	38.8 ± .5

* Ip = Iproniazid; Is = Isoniazid.

tivity of ephedrine and a-methylphenylethylamine should not be potentiated by iproniazid. The results again are entirely consistent with this assumption. Furthermore, isoniazid, which inhibits MO much less than iproniazid(1,5) is also a less effective potentiator of the action of phenylethylamine. There is also a clear indication that the toxicity of tyramine, another substrate of MO, is increased by the pretreatment of the guinea pig with iproniazid.

The results obtained on non-anesthetized animals are in perfect accordance with those found in an entirely different experimental system. The response of the nictitating membrane of anesthetized cats to phenylethylamine and tyramine was enormously potentiated by iproniazid, but not by isoniazid(5).

Objections may be raised against the assumption of identity of the symptoms produced by phenylethylamine and ephedrine. The non-toxic dose of 12 micromoles per g body weight of phenylethylamine intensified the response to ephedrine by at least one degree, indicating that both amines may act on the same receptors.

The question arose whether or not the symptoms described here are related to the syndrome produced by the administration of semicarbazide and other carbonyl reagents(6). Since pyridoxine reduces the toxicity of semicarbazide, this vitamin was tested also against phenylethylamine. The administration of 144 micromoles of pyridoxine 1 hour prior to phenylethylamine did not prevent the usual neurological response either in untreated guinea pigs (0.9 micromole per g body weight) nor in animals pretreated with ironiazad (0.09 micromole per g body weight).

The onset of the toxic syndrome described above is accompanied by a marked increase of the body temperature. In this respect the combination of phenylethylamine plus 350 micromoles of isoniazid is almost as effective as phenylethylamine plus 150 micromoles of iproniazid. Since ephedrine is not a substrate of MO, one result remains unexplained: The decrease in temperature, observed after the administration of ephedrine alone, seems to be reversed when the animals are pretreated with iproniazid (probability$<$0.05). The difference, however, is much smaller than for the system phenylethylamine/iproniazid (probability $<<$0.01).

Conclusions. The toxic action of phenylethylamine and tyramine, but not of ephedrine and D-a-methylphenylethylamine is increased by pretreating guinea pigs with iproniazid. Isoniazid is less effective. The results are explained by the blocking of MO by iproniazid.

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Association of Polonium-210 with Blood. (21340)

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The importance of blood in the metabolism of polonium has not been thoroughly investigated. However, certain observations made on polonium-intoxicated animals, such as: prolonged elevation of blood polonium after injection(1,2); pathological alteration of several organs such as spleen, bone marrow and liver(3,4); polonium induced anemia(5); and biliary excretion of polonium(1,6), suggest the importance of blood in the polonium toxicity syndrome.

The purpose of this study was to make a detailed investigation into the distribution of polonium in the blood of rats and dogs.

Method. Most of the work reported herein was performed with adult male rats of the Sprague-Dawley strain, weighing 250 to 300 g. These rats were housed in colony cages, and fed a commercial dog chow and water *ad lib.* Blood samples were obtained by cardiac puncture from Nembutal anesthetized animals. The blood was taken 4 days after the animals had been intravenously injected with approximately 50 μc of polonium per kilo body weight. It has been shown that this time interval is required for the polonium blood level to become relatively constant(2). Critical experiments were repeated using mongrel dogs instead of rats, with almost identical results. The procedure described here for the determination of polonium is a modification of that of Fink *et al.*(1). Samples were wet-digested with nitric and perchloric acids, and the resulting solution was neutralized, then diluted to a standard volume with 0.5 N HCl. The polonium was plated in 2 stages from duplicate aliquots of this solution on copper discs. The radioactivity of the discs was measured in an alpha counter. If agreement within 5% between duplicates was not obtained additional aliquots were analyzed. This method has been found to be accurate within a standard deviation of ± 5%. The major formed elements of blood were isolated by centrifugation. Auxiliary experiments were performed which showed that the technics used, such as washing the cells with isotonic NaCl, had no effect on the distribution pattern. In the chemical fractionation of whole blood the method of Grinstein(7) was used for the isolation of hemoglobin. Several methods were used for the separation of heme and globin, but the only one which appeared to be satisfactory was a continuous extraction of hemoglobin powder with methanol(8). Measurement of the affinity of certain blood components for polonium was made by determining the distribution of polonium between the solution being investigated and an ion exchange resin. The system used for these studies consisted of the following: 100 mg of IR-112*H acid-charged cation exchange resin (washed free of excess acid): 20 ml of the test solution *i.e.*, water or blood cell component); and 5 ml of a polonium solution having 2,000 counts per minute per ml. The reactants were placed in 125 ml glass-stoppered Erlenmeyer flasks, adjusted to pH 6.8 (1 or 2 drops of 0.1 N NaOH), and shaken for approximately one hour at 4°C. Five milliliter aliquots were then assayed for polonium. The distribution constant (K_d) for each test system was calculated by the method of Schubert(9). The native globin used in these studies was prepared by the method of Drabkin(10).

Results. The polonium content of the blood components which were obtained by centrifu-

TABLE I. Polonium Content of Centrifugally Separated Blood Components (Expressed as % of the Amount Found in Whole Blood Sample).

Component	Avg % retained rat blood	Observed range	Avg % retained dog blood	Observed range
Plasma	5	3- 6	4	3- 5
Red blood cells	92	67-97	88	85-92
White blood cells	4	2- 5	6	5- 6

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* Mound Laboratory is operated by Monsanto Chemical Co. for the U. S. Atomic Energy Commission under Contract No. AT-33-1-GEN-53.

TABLE II. Polonium Content of Chemically Separated Components of Whole Blood from Polonium-Injected Rats.*

Isolated component	Polonium content % of whole blood	 Observed content
Hemoglobin	96	89 -101
Heme	.2	.17- .26
Globin	74	70 - 79

* Values based on avg of 3 experiments.

gal fractionation of whole blood from polonium-injected rats and dogs is tabulated in Table I. It is seen that most of the polonium in whole blood was associated with the red blood cells, while a negligible amount was found with the plasma or white blood cells. Although the total amount of polonium in the white blood cells was very small, the specific activity of these cells was found to be approximately the same as that of the red blood cells.

Table II presents a summary of the results obtained from the chemical fractionation of whole blood from polonium-injected rats. The majority of the polonium was found to be in the hemoglobin. When the hemoglobin was separated into its component parts almost all of the polonium was found with the globin portion and practically none was found with the heme. The procedure for the separation of heme and globin described herein was found to have no directing effect on the localization of polonium as was observed in other separation schemes. For example, if the separation was carried out under acidic conditions the polonium remained in solution; conversely, under alkaline conditions the polonium would precipitate with the material being separated. It is clear from these observations that chemical separations which rely on or involve a change in hydrogen ion concentration would be unsatisfactory for this type of study.

From results obtained by starch column electrophoresis studies with hemoglobin and plasma solutions, Thomas and Stannard(11) have reported the association of polonium with hemoglobin. The validity of further fractionation studies by them which indicated that polonium was associated with the globin would appear to be subject to criticism since the heme was isolated as the protoporphyrin dimethyl ester. In this isolation changes in hydrogen ion concentration would normally be employed.

In order to verify the association of polonium with the globin portion of red blood cells, measurements were made of the strength of the bonds between polonium and various components of red blood cells. The method consisted of comparing the relative affinity for polonium of: water; a lysed red blood cell solution made from the blood of normal rats (1 ml whole blood diluted to 1000 ml water); a solution of native globin; and a heme residue of equivalent concentration prepared from the same blood. The results of this study, presented in Table III, show that native globin and lysed red blood cells have the same affinity for polonium, while heme has approximately the same affinity as that of water. These results indicate that globin would be the location of polonium in red blood cells.

Summary. 1. When whole blood from polonium-injected rats or dogs was centrifugally fractionated, it was found that approximately 90% of the polonium was associated with the red blood cells. 2. Isolation of hemoglobin from similar blood indicated that the polonium was associated with this portion of the red blood cell. Fractionation of hemoglobin into globin and heme indicated that almost all of the polonium was associated with the globin. Measurement of the affinity of major red blood cell components for polonium demonstrated that globin had as much affinity as lysed whole red cells, while heme residue had practically none. 3. Chemical methods which rely on a change in hydrogen ion concentration for the

TABLE III. Comparative Affinity of Water, Lysed Whole Blood, Native Globin and Acid Hematin for Polonium.*

Material tested	Avg k_d†	K_dH_2O/K_d test sol.
Water	778	1
Lysed whole blood	62	12
Native globin	62	12
Acid hematin (acetone)	674	1.1

* Values based on avg of 3 experiments.

$$\dagger\ K_d = \frac{\left(\begin{array}{c}100 - \%\text{ polonium}\\ \text{in supernatant}\end{array}\right)}{\left(\begin{array}{c}\%\text{ polonium}\\ \text{in supernatant}\end{array}\right)} \cdot \frac{\text{Vol of sol.}}{\text{Dry wt of resin}}$$

isolation of polonium containing red blood cell components were not satisfactory.

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Serial Passage of Human Tumors in Chick Embryo: Growth Inhibition by Nitrogen Mustard.* (21341)

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Murphy(1) first reported successful growth and serial passage of rat and mouse tumors on the chorioallantoic membrane (CAM) of the chick embryo. Murphy(2) and others(3-5), however, were unable to maintain human tumors by serial transfer on the CAM; although some tumor cells apparently survived and showed mitotic figures during the initial explantation, tumor growth, when it occurred, was slow. Toolan(6) demonstrated that certain human tumors, when transplanted into rats pre-treated with x-rays, survived and showed active cell multiplication. Subsequently she found(7) that use of rats or hamsters conditioned with cortisone, with or without x-rays, resulted in increased numbers of successful transplants, associated with rapid growth and prolonged survival of the tumor. Approximately 2 to 3% of explanted human tumors showed a high and sustained rate of growth, and these have been maintained by serial passage in properly conditioned rats and hamsters.

Three of these human tumors have been explanted to the CAM and the yolk sac of the chick embryo; their survival and growth characteristics on continuous transfer are presented in this report. In addition, 2 of the tumors, growing on the CAM, were treated with methyl-bis (β-chloroethyl) amine hydrochloride, (HN2), a nitrogen mustard, and responded in a manner similar to that of the mouse sarcoma 180 reported earlier(8,9).

Materials and methods. The tumors are the Human Sarcoma #1 (H.S. #1), Human Epidermoid Carcinoma #3, (H. Ep. #3), and Human Embryonal Rhabdomyosarcoma #1 (H. Emb. Rh. #1). Detailed descriptions of these tumors have been given elsewhere(10). *Fertile White Leghorn eggs,* incubated at 38° were used. Explants to CAM were made with single fragments of tumor on 7th to 9th day of incubation. On 16th to 19th day the embryo

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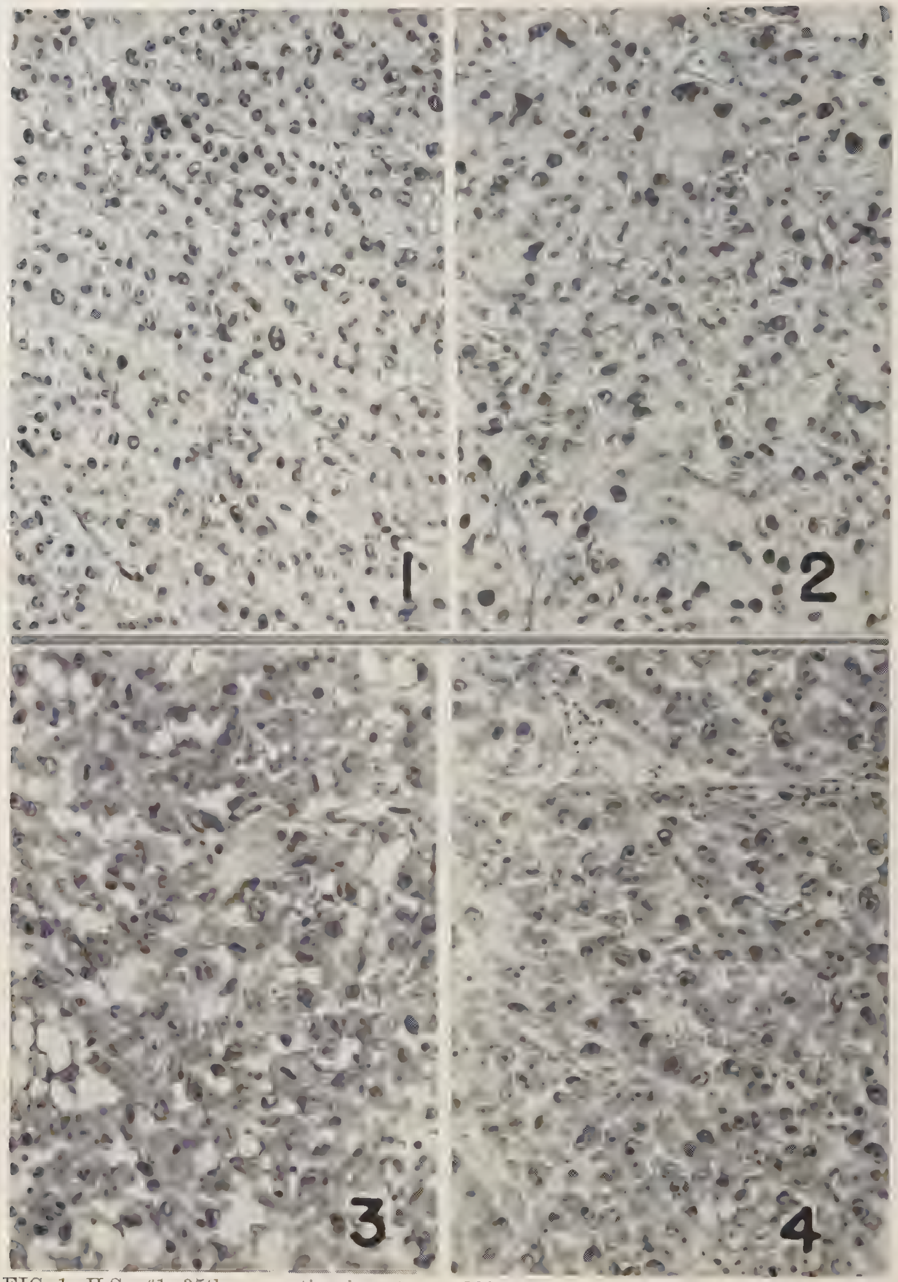
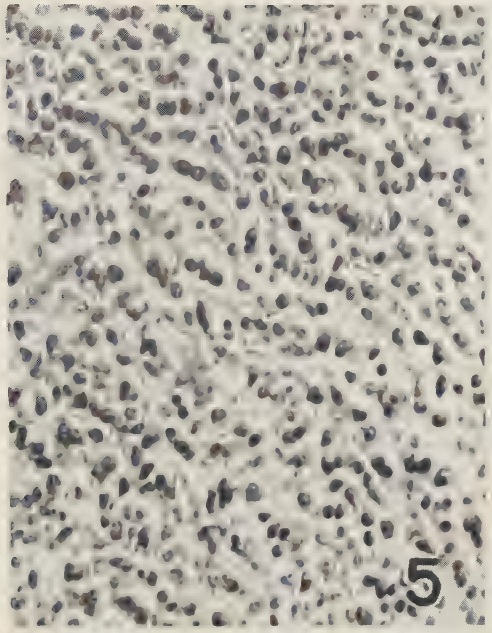
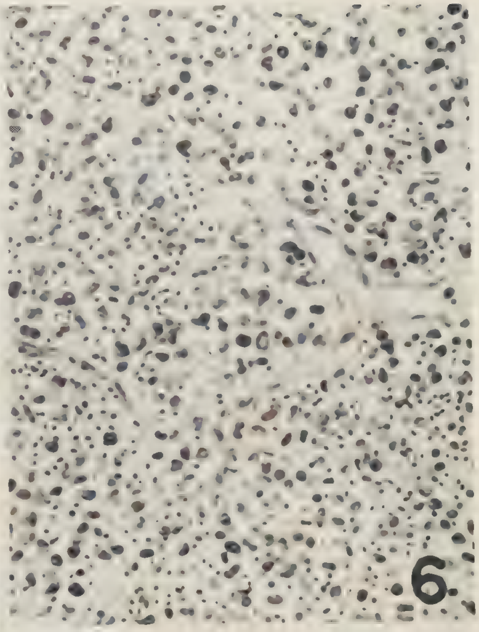
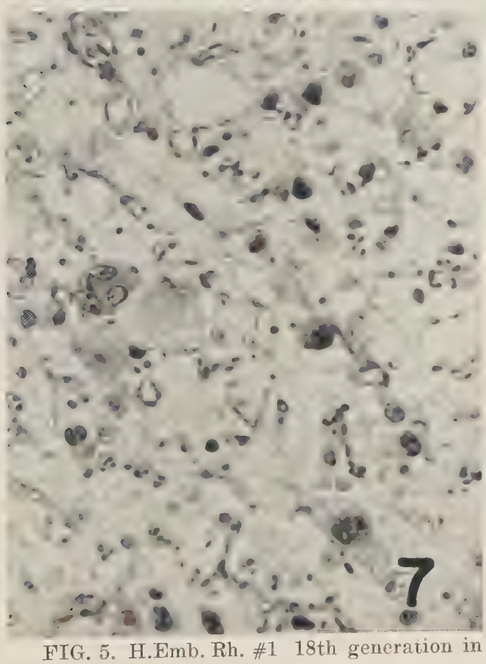


FIG. 1. H.S. #1 35th generation in rats. × 200.
FIG. 2. " 6th " on the CAM. × 200.
FIG. 3. H.Ep. #3 11th " in hamsters. × 200.
FIG. 4. " 3rd " on the CAM. × 200.

was sacrificed, the tumor described grossly, weighed, and a portion prepared for microscopic examination. The largest tumors were cut into fragments and explanted to other eggs. Occasionally, the liver, metanephroi, lungs, brain and spleen were fixed and examined microscopically for metastases. *Yolk sac* inoculations of 0.1 to 0.2 cc of a 10 to 20% suspension of the H.S. #1 and H. Ep. #3 in saline were made on 4th day of incubation. Embryos were sacrificed on 16th to 18th day and treated in the same manner as the CAM explants.







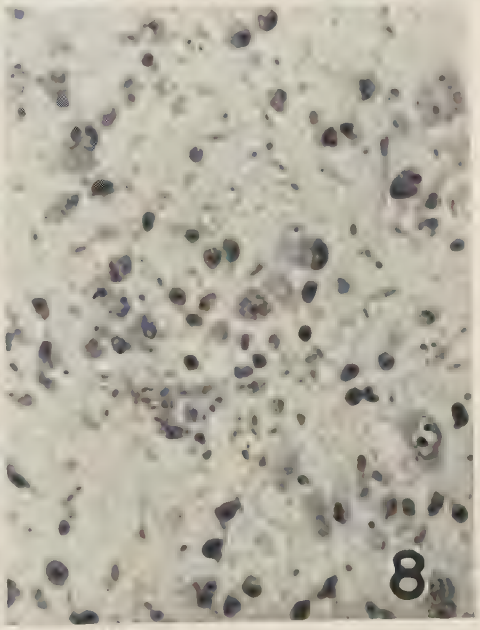


FIG. 5. H.Emb. Rh. #1 18th generation in rats. × 200.
FIG. 6. " 1st " on the CAM. × 200.
FIG. 7. H.S. #1 6th " " " ".
HN2 injected into yolk sac. × 400.
FIG. 8. H.Ep. #3 1st generation on the CAM.
HN2 injected into yolk sac. × 400.

HN2, dissolved in saline, was tested against H.S. #1 and H. Ep. #3 growing on the CAM. On 12th day of incubation, 4 days after tumor had been inoculated, 0.1 mg of HN2 (0.1 cc) was injected into the yolk sac. Four to 6 days later the embryo was sacrificed; the tumor was removed and weighed, and portions of the tumor preserved for microscopic examination.

Results. Human Sarcoma #1: This tumor grew rapidly on CAM and retained its growth rate after 8 continuous passages in the egg. When removed from the CAM, 8 to 10 days after explantation, the median weight for 52 tumors was 140 mg; approximately 50% of the recovered specimens weighed between 100 and 600 mg with occasional ones as low as 10 mg and one large tumor of 4 g. The tumors were solid and necrotic areas rare. Of a total of 248 eggs with CAM explants of H.S. #1, 62% (155) of the eggs survived until day of sacrifice, and 59% (92) of survivors had tumors. *Yolk sac inoculation* of the H.S. #1 has been attempted on 3 occasions. A total of 25 eggs were used; 15 of these survived until the day of sacrifice, and 6 of the survivors had tumors. The average weight of the yolk sac tumors was approximately 50 mg.

The rat grown H.S. #1 by microscopic examination is composed of large cells with foamy pale cytoplasm and of some smaller more densely staining cells. There are numerous mitotic figures and hyperploid giant cells are frequent. These characteristics are shown in Fig. 1 which is a section obtained from 35th transplant generation in rats from which the original chick embryo explantation was derived. Histological appearance of the tumor was largely retained during growth in the chick embryo after CAM and yolk sac inoculation. Giant cells were found in all passages, and the tumor was well vascularized (Fig. 2) HS #1 cells are larger in the egg than in the rat host; a similar situation was observed in cells of sarcoma 180 when grown in the egg.

Embryos carrying H. S. #1 on the CAM or yolk sac did not show any consistent inhibition of growth or evidences of toxicity. Microscopic examination of livers, lungs, kidneys, and spleens from several embryos has not revealed the presence of metastatic cells. H.S. #1, after the 4th egg generation, was reimplanted into conditioned rats. The tumor grew as well as those which had been continuously carried in these hosts.

Human Epidermoid Carcinoma #3: This tumor has been carried through 6 generations on the chorioallantois. Since the 3rd generation on CAM it has been carried in the yolk sac, also. Tumors recovered from the CAM weighed between 10 and 1270 mg, median weight 230 mg for 75 tumors. Seventy-five per cent of tumors fell between 150 and 790 mg. Following 186 explants to the CAM, 91% of the eggs (169) survived until the time of sacrifice on 16th to 19th day of incubation, and 70% (118) of these survivors had tumors. The larger tumors, usually those exceeding 400 mg often had necrotic centers. Eighty-eight implants were made into the yolk sac; 35% (31) survived and 87% (27) of these had tumors. Stained microscopic preparations show that cells of the hamster-grown H. Ep. #3 are relatively uniform in size and shape with a homogeneous cytoplasm (Fig. 3). These characteristics persisted after explantation to the CAM and yolk sac. (Fig. 4). In the egg, tumor cells were closely packed, but there were occasional isolated groups of cells at the edges of the tumor growing on the CAM. Sometimes blood vessels on the CAM were seen to contain tumor cells, and there were frequent metastases in the liver after both CAM and yolk sac inoculation. Metastatic cells have been seen in the metanephroi of 2 chicks, but, so far, none has been found in the lung, brain or spleen. After 2 generations in the egg, H. Ep. #3 was inoculated into conditioned rats, and it showed its usual rapid growth.

Human embryonic rhabdomyosarcoma #1. The H. Emb. Rh. #1 has been maintained for 3 generations on the CAM. Thirty-three implants have been made; 64% of these survived (21) and 48%(10) of the survivors had tumors. The tumors weighed between 60 and 615 mg, average 280 mg, for 10 tumors. In the rat the tumor is composed of small, darkly-staining cells (Fig. 5), and this histological appearance is retained in the chick embryo (Fig. 6). The tumor induces a marked proliferation of blood vessels, and it is highly vascular, hemorrhagic and friable in the egg, as well as in the rat.

Results with Nitrogen Mustard: HN2, 0.1 mg/egg, induced some degree of histological injury in the H. S. #1 and the H. Ep. #3. The drug was injected into the yolk sac at 12 days, and when tumor was removed 4 to 6 days later, it was solid and appeared to be smaller in size. Microscopically, the H.S. #1 was not consistently affected at this dose, although in some tumors a marked decrease in cellularity was noted (Fig. 7). H. Ep. #3 showed a similar response to HN2 in most cases, and the remaining tumor cells were enlarged, with nuclear abnormalities (Fig. 8). There was considerable interstitial connective tissue and a myxomatous matrix between tumor cells. The chick embryos showed no gross abnormalities at this dose.

Discussion. These tumors have retained their human specificity during prolonged heterologous transplantation. Korngold(11) has demonstrated, by serological methods, that the H.S. #1 and H. Ep. #3 after many continuous passages in the rat and egg have retained human tumor tissue antigens. In a preliminary study, Denues and Mottram(12) examined the metaphase chromosomes of egg-grown H. Ep. #3 and found that the large cells of this tumor have characteristic human chromosomal complement, quite distinct from avian or rodent material.

H.S. # 1, H. Ep. #3 and H. Emb. Rh. #1 fall into what appears to be a special group of tumors, which exhibit continuous and rapid growth in conditioned rats and hamsters and chick embryos. Although it is possible that these tumors would have shown continuous growth if they had been transplanted directly from the patient to the chick embryo, the cortisone-treated rat appears to have some advantage over the chick embryo for selection of rapidly growing, transplantable tumors. Repeated experiments have shown that human tumors or embryonic tissues planted directly in tissue culture do not do as well as rat-grown material of the same tissues(13).

While many previous attempts to explant human tumors directly to fertile eggs have not resulted in tumors which could be maintained by continuous egg passage, it should also be noted that only a small percentage of human tumors transplanted to conditioned rats and hamsters(8) have survived continuous passage.

Results obtained with HN2 against the H. S. #1 and H.Ep #3 are similar to those seen with the same drug against the sarcoma 180, in that there is a destruction of a large number of tumor cells with enlargement and nuclear changes in surviving cells at doses not lethal to the embryo. While adequate quantitative data are not yet available, human tumors appear to be somewhat more resistant than sarcoma 180 to HN2. A single dose of 0.05-0.1 mg/egg(8,9) had a marked destructive effect on sarcoma 180, whereas 0.1 mg/egg produced a similar degree of injury in the H.Ep. #3, and a less consistent effect on the H.S. #1. It is of interest that the pattern of histological response of human tumors to HN2 closely resembles that of mouse sarcoma 180.

Summary. Human Sarcoma #1 (H.S. #1), Human Epidermoid Carcinoma #3 (H.Ep. #3) and Human Embryonal Rhabdomyosarcoma #1 (H. Emb. Rh. #1) have been grown by serial passage on the chorioallantoic membrane of the chick embryo, and the H.S. #1 and H.Ep. #3 by serial passage in the yolk sac. Metastases of the H.Ep. #3 to the liver and kidneys of the host chick have been observed. The H.S. #1 and H. Ep. #3, when treated with a nitrogen mustard, HN2, showed the same pattern of histological injury previously described for the mouse sarcoma 180 growing in chick embryos and mice.

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Factors Influencing Conversion of Folic Acid to Citrovorum Factor by Chick Liver Fractions.* (21342)

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The presence in rat and chick liver of an enzyme system converting pteroylglutamic acid (PGA) to citrovorum factor (CF) has been reported(1-5). The importance of this enzyme system has become evident in recent years in that reports have appeared indicating a probable CF-catalyzed utilization of formate in the synthesis of purines(6,7). Nichol and Welch(1) reported that ascorbic acid augments the formation of CF from PGA by rat liver slices. Up to 25% of the added PGA is converted to CF by homogenates of chick liver incubated anaerobically in the presence of ascorbate(2). Addition of homocysteine gives a 50-60% increase in the conversion of PGA to CF by chick liver homogenate and under these conditions, glutathione, ascorbic acid and cysteine are without effect(3,4). Supplementation of chick diet either by vit. B_{12} or PGA results in an increased capacity by the liver homogenate to convert PGA to CF(5). Broquist *et al.*(8) reported a 39-54% conversion of PGA to CF when the resting cells of *Streptococcus faecalis* A(9) were incubated with PGA and a supply of "formate" in the presence of reducing agents or in an atmosphere of nitrogen. Cell free extracts of *S. faecalis* A require addition of glucose, ascorbate, formate, Mg^{++}, DPN and ATP for optimal formation of CF from added PGA(10).

The present report concerns the study of factors affecting PGA to CF conversion by the chick liver fractions.

Methods. Straight run (New Hampshire males × Single Comb White Leghorn female) crossbred chicks of 4-5 weeks of age were used in studying the effect of reducing agents in the *in vitro* conversion of PGA to CF. The diet used consisted of a folic acid deficient diet (Nutritional Biochemical Corp., Cleveland 28, Ohio) supplemented by 4 mg PGA, 3.5 g DL methionine, and 20 g cod liver oil U.S.P. (850 units vit. A and 85 units vit. D/g) per kg of diet. At the end of the test period, chicks were sacrificed, the livers were removed and immediately tested for PGA to CF conversion studies as described earlier(5). Livers from straight run crossbred chicks (New Hampshire males × Single Comb White Leghorn female) of 10-12 weeks of age were used for fractionation of the PGA to CF converting enzyme. Birds were fed Purina Startena (Ralston Purina Co.) beginning one day of age. The chick liver fractions were prepared in the following manner: 20% homogenates of chick liver were made in 0.08 M sodium potassium phosphate buffer pH 6.3, using a Waring blendor. An aliquot of the homogenate was centrifuged for 30 minutes at 13000 x g and the clear portion of the supernatant was decanted off and made to the volume of the starting homogenate with an aliquot of the same buffer. The residue was also made to the volume of the original homogenate by addition of the buffer. Five ml each of the 20% homogenate, of the supernatant fraction and of the residue were tested for PGA to CF conversion studies as described earlier(5). An aliquot of the supernatant was dialyzed against distilled water for 7 days and the protein fraction was made to a volume corresponding to 200 mg/ml of chick liver by addition of the 0.08 M phosphate buffer. Chick liver supernatant was also precipitated with ethanol at 15% concentration and the mixture centrifuged. The precipitated material was made to the same volume as the starting homogenate. To the centrifugate was added enough alcohol to give a 40% final concentration. The mixture was re-centrifuged and the precipitated material was made to a volume equivalent to 200 mg/ml of the starting liver. 5 ml aliquots of the undialyzed protein fraction and of the 15% and 40% alcohol precipitated material were tested for PGA to CF conversion by the previously described procedure(5). Chick liver acetone powders were made in the following manner: To the freshly chilled chick livers were added 2 volumes of ice water and homogenized in a Waring Blendor. The homogenate was squeezed through muslin and to the filtrate were added 2 volumes of ice cold acetone. The acetone precipitated material was immediately filtered on Buchner funnel with suction, and the residue was washed with acetone and ether. The cake of the acetone powder was dried under vacuum over P_2O_5. A weighed portion of the acetone powder equivalent to 25 g of the starting chick liver was reconstituted in 125 ml of the 0.08 M sodium potassium phosphate buffer and aliquots were tested immediately for PGA to CF conversion studies. All samples were incubated for 2 hours under N_2. The CF content of the liver samples was determined using the Bacto-CF Assay Medium (Difco Laboratories, Inc., Detroit 1, Michigan). *Leuconostoc citrovorum* ATCC 8081[†] was used as the test organism, and Leucovorin (Lederle) was employed as the standard. The cultures were incubated 48 hours at 37°C and growth was measured by turbidimetric procedure. Since Leucovorin has been reported to be half as active as the CF isolated from a horse liver(11), the microbiological assay values were divided by 2 in order to express the results in terms of the naturally occurring CF.

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Results. It is apparent from the results of Table I that the addition of 5 mg homocysteine gives a 50% increase in the conversion of PGA to CF by the chick liver homogenate. Under these conditions 5 mg each of homocystine, cystine and glutathione, or 10 mg ascorbic acid are without effect. On the other hand 5 mg cysteine inhibits the conversion of PGA to CF. Thus the stimulatory effect of homocysteine on CF synthesis is specific since other sulfhydryl as well as nonsulfhydryl compounds are without effect.

TABLE I. Influence of Reducing Agents on PGA to CF Conversion by Chick Liver Homogenate.

Substances added/flask*		γ CF/g liver homogenate
	mg	
None		6.4
PGA, 100 γ		40.5
" + homocysteine	5	62.5
" + homocystine	5	46.8
" + cysteine	5	15.8
" + cystine	5	44.0
" + glutathione	5	42.4
" + ascorbic acid (pH 6.3)	10	40.0

* Flask components: 5 ml of 20% liver homogenate in 0.08 M sodium potassium phosphate buffer pH 6.3 along with 5 ml of the same buffer and aliquots of solutions containing substances described to make a total volume of 11 ml. Flasks were incubated for 2 hr under N_2.

The results on the effect of added formate, serine, homocysteine, Mg^{++}, ATP and DPN on PGA to CF conversion by chick liver fractions are presented in Table II. Homocysteine consistently enhances the PGA to CF conversion by all the chick liver fractions tested. Addition of Mg^{++} and glycine inhibits the PGA to CF conversion by the chick liver homogenate while serine enhances the conversion. The stimulatory effect of homocysteine on CF synthesis by the liver homogenate is inhibited by formate and by Mg^{++}. On the other hand addition of homocysteine or Mg^{++} but not of formate or serine increases the PGA to CF conversion by the chick liver supernatants. Such increase in PGA to CF conversion by the supernatant was further enhanced by addition of homocysteine, Mg^{++} and serine together. Addition of formate, serine, homocysteine or Mg^{++} enhances the PGA to CF conversion by acetone powders of chick liver. Optimal formation of CF by the acetone powders of chick liver requires addition of serine, homocysteine and Mg^{++}. Under these conditions formate, cysteine and Cu^{++} have been found ineffective in duplicating the effect of serine, homocysteine and Mg^{++} respectively. Conversion of PGA to CF by the undialyzed protein fraction and by the 40% alcohol precipitated chick liver fraction is apparently optimal

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[†] A recent report (Felton, E. A., and Niven, C. F., *J. Bact.*, 1953, v65, 482) concerning a taxonomic study of the culture *Leuconostoc citrovorum* ATCC 8081 suggests that the latter organism is a typical strain of *Pedicoccus cerevisiae* as described earlier by Pederson (*Bact. Rev.* 1949, v13, 225).

TABLE II. Effect of Formate, Serine, Homocysteine, Mg^{++}, ATP and DPN on PGA to CF Conversion by Chick Liver Fractions.

Substances added/flask*	Chick liver fractions† 1	2	3	4	5
	γ CF/g			mγ CF/g	
None	6.3	.2	1.7	50	30
PGA	33.3	12.7	1.9	53	28
” + F‡	36.3	10.7	3.4	55	25
” + S	41.8	11.4	3.9	55	25
” + H	45.0	18.7	10.7	163	50
” + Mg^{++}	16.6	18.8	6.4	55	33
” + F + H	35.6	17.2	9.3	156	53
” + S + H	56.8	19.0	14.6	203	80
” + S + H + Mg^{++}	37.8	43.3	30.2	234	110
” + S + H + Mg^{++} + DPN + ATP	—	—	—	280	225

* Flask components: 5 ml of 20% liver homogenate or an equivalent amount of the chick liver fractions in 0.08 M sodium potassium phosphate buffer pH 6.3 and aliquots of solutions containing the following amounts of various substances to make a total volume of 11 ml: PGA, 100 γ; formate, 10 mg; serine, 10 mg; homocysteine, 5 mg; magnesium chloride, 10 mg; DPN (90% pure, Sigma Chemical Co., St. Louis, Mo.), 2 mg; and ATP (95% pure disodium salt, Nutritional Biochemical Co., Cleveland 28, Ohio), 1 mg. Flasks were incubated for 2 hr under N_2.

† Chick liver fractions 1 to 5 are as follows: homogenate, supernatant, acetone powder, 40% alcohol precipitated material, and undialyzed protein fraction respectively.

‡ F = Formate; S = Serine; H = Homocysteine.

in presence of serine, homocysteine, Mg^{++}, ATP and DPN. On the other hand the chick liver residue and the 15% alcohol precipitated chick liver fraction give only a slight PGA to CF conversion even after addition of serine, homocysteine, Mg^{++}, ATP and DPN.

Discussion. The components required for the optimal conversion of PGA to CF by cell free extracts of *Streptococcus faecalis* A include PGA, glucose, ascorbate, formate, Mg^{++}, DPN and ATP(10). Under the presently described experimental conditions homocysteine specifically increases the conversion of PGA to CF by the chick liver fractions. Berg(12) reported that in a pigeon liver extract system homocysteine specifically enhances the incorporation of formate into serine, purines and methyl group of methionine probably by forming a homocysteine-C_1 compound which may be a common precursor in one carbon transfers. Since the *in vitro* synthesis of CF from PGA also involves a transformylation reaction, it may be possible that homocysteine is in some way enhancing the utilization of one carbon fragments in the synthesis of CF. Involvement of serine in PGA to CF conversion may be indicated by the report(13) that tetrahydrofolic acid is required for the interconversion of glycine to serine by Dowex-1 treated pigeon liver extracts. In the present studies glycine determinations(14) were made on the chick liver acetone powder samples prepared for CF assay. It was observed that addition of either serine, Mg^{++}, or homocysteine to the acetone powder of chick liver give a 2-fold increase in the glycine content while a 4-fold increase in the glycine content of the acetone powder was obtained when serine, Mg^{++} and homocysteine were added together. This would indicate that the experimental conditions which are suitable for an optimum CF synthesis also results in an increased glycine formation. The effect of added formate in decreasing the stimulatory effect of homocysteine on CF synthesis by the liver homogenate and that of glycine in inhibiting the conversion may be due to a retardation of endogenous serine utilization for CF synthesis. The results on PGA to CF conversion by the alcohol precipitated material or by the undialyzed protein fraction indicate a considerable loss in the converting enzyme activity. In order to ascertain if this decrease in the enzyme activity was partly due to the removal in the dialysate or in the 40% alcohol soluble fraction of one or more factors other than serine, homocysteine, Mg^{++}, ATP and DPN which are essential for optimal conversion, studies on the effect of added boiled chick liver juice on the conversion were conducted. It was observed that the addition of boiled chick liver juice to the alcohol precipitated fraction gives a 3-fold increase in the maximum obtainable conversion while under these conditions a 2-fold increase in PGA to CF conversion was obtained by addition of the boiled chick liver juice to the undialyzed protein fraction. This would indicate that one or more unidentified factors present in the chick liver homogenate are essential for an optimal PGA to CF conversion by these fractions.

Summary. The supernatant fraction of chick liver is the principal source of the PGA to CF converting enzyme system. The latter is precipitated by 40% alcohol. Homocysteine consistently enhances PGA to CF conversion by the chick liver fractions. The enzymatic synthesis of CF from PGA by acetone powders of chick liver is optimal in presence of homocysteine, serine, and Mg^{++}. Under these conditions the glycine content of the acetone powder is increased 4-fold.

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Effect of Total Body X-Irradiation on Desoxyribonuclease Activity of Rat Spleen.* (21343)

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In view of the effects of relatively small doses of total body x-irradiation upon the desoxyribonucleic acid (DNA) content of various tissues, particularly lymphoid tissue(1-4), it was of interest to determine the effect of this type of x-irradiation on desoxyribonuclease (DNAase). Carter(5) was unable to demonstrate an effect of total-body irradiation on this enzyme. Recently Cunningham and Laskowski(6) have found that there are two enzymes in tissues with different pH optima which bring about the depolymerization of DNA. Kowlessar, *et al.*(7) have observed the appearance of DNAase activity in the urine of rats after total-body irradiation. Eichel and Roth(8) observed no effect on the enzyme in *Tetrahymena geleii* W after the organisms had been subjected to 500,000 r.

We have measured the DNAase activities at pH 5 and 7.4 in the livers and spleens from rats receiving sublethal doses of x-irradiation and have observed increased activity at pH 5. A dose-response experiment has also been done.

Experimental. Sprague-Dawley rats were used. In certain experiments adult rats of both sexes were used and in others 100-150 g males. The animals were fed a diet of Purina laboratory chow and were given food and water *ad libitum.* The animals were irradiated

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TABLE I. Effect of 500 r of Total Body X-Irradiation on Average DNAase Activities[*] of Rat Tissues Measured by the Method of Schneider and Hogeboom(9).

Time when animals were sacrificed after x-ray	Liver pH 5	Liver pH 7.4	Spleen pH 5	Spleen pH 7.4
Control (6)	1.19 ± .08[†]	5.54 ± 1.02	4.06 ± .16	3.02 ± .17
12-24 hr (11)	1.19 ± .16	6.60 ± .83	6.12 ± .34	3.28 ± .29
48 hr (6)	1.20 ± .19	7.26 ± .64	5.04 ± .33	2.52 ± .39

[*] Expressed as (units of optical density at 260 mμ/mg nitrogen) × 10.
[†] Stand. error.
Numbers in parentheses refer to No. of animals used.

using the following physical factors: 220 KV, 15 ma, inherent filtration only, HVL 0.25 mm. Cu, 32 inches to the center of the animal, field size covering the entire animal and at a rate of 35.5 r per minute in air. The animals were killed by stunning and decapitation after having lived for various lengths of time after irradiation. In preliminary experiments DNAase activities were measured by a method similar to that of Schneider and Hogeboom (9). Modifications included increasing the incubation time to 3 hours and centrifuging, after perchloric acid precipitation for 20 minutes at high speed in the cold. The "neutral" enzyme was determined in the presence of 0.01 M phosphate buffer pH 7.4 and 0.01 M MgSO$_4$. The "acid" enzyme was determined in 0.01 M succinate buffer pH 5 in the absence of added Mg^{++}. The substrate in these cases was a commercially available highly polymerized DNA preparation.[†] Later determinations were carried out by the method of Laskowski(10), the substrate being again a highly polymerized commercially available preparation.[‡] Nitrogen was determined by the Koch procedure(11).

TABLE II. Effect of 500 r of Total-Body X-Irradiation on Average DNAase (pH 5) Activities of Rat Spleen as Measured by the Method of Laskowski(10).

Time when animals were sacrificed after x-ray		μg P/mg N/hr
Control	(9)	8.1 ± .7[*]
10 min.	(3)	8.2 ± 1.1
2.5 hr	(1)	7.2
24 hr	(7)	20.7 ± 1.9

[*] Stand. error.
Numbers in parentheses refer to No. of animals used.

Results. Results of initial experiments in which the DNAase activities were determined by the method of Schneider and Hogeboom (9) are presented in Table I. Measurement of the production of acid soluble phosphorus(10) gave results in Table II. In the measurements of Carter(3) an unbuffered homogenate of spleen was used. Assuming that the pH's of these homogenates were near neutrality, it is not surprising that no change on irradiation was observed in the light of the results presented here. It is seen that the activity at pH 7.4 in spleen is not appreciably affected by x-irradiation while that at pH 5 is significantly altered. The slight activity of liver at pH 5 is not changed by the x-ray treatment. It is possible that this activity is not due to the "acid DNAase" but rather to "neutral" enzyme acting suboptimally. The application of the "t" test(12) yields a P value for the change in activity at pH 7.4 in liver of > 0.1 and for that in spleen at pH 5 of <0.01.

There is the possibility that the increase in activity of the "acid DNAase" may be due to a change in the cellular population of the spleen. Murray(13) observed that virtually all the lymphocytes of spleen were destroyed in from 1 to 3 hours post-irradiation. It appears likely that in the cellular disorganization which follows this that the enzyme may be released from bound forms and hence the apparent activity would become greater. It is seen from Table II that the time of maximum activity after irradiation corresponds well with the time at which reticular cells become promi-

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[†] Purchased from Worthington Biochemical Sales Co., Freehold, New Jersey.

[‡] Purchased from California Foundation for Biochemical Research, Los Angeles, Calif.

TABLE III. Response of DNAase (pH 5) of Rat Spleen to Increasing Doses of Total-Body X-Irradiation.

Dose	Avg activity (μg P/mg N/hr)
0 r (9)	8.1 ± .7
100 r (3)	17.5 ± 4.8
200 r (3)	24.4 ± 2.8
500 r (7)	20.7 ± 1.9

Numbers in parentheses refer to No. of animals used.

nent in the white pulp(13). It is during this time (24-48 hours post-irradiation) that the DNA content of the spleen is at its minimum (14).

Table III shows that a maximal effect on the enzyme is produced at a dose of 200 r.

Summary. 1. The "acid-active" DNAase of rat spleen has been found to increase markedly after 500 r of total body x-irradiation. 2. Such a change is not apparent in the "neutral" enzyme of liver or spleen. 3. The maximum in activity of the elevated DNAase occurs 24-48 hours post-irradiation. 4. Maximal response is elicited at a dose of 200 r.

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Cardiovascular and Central Nervous System Effects of Morphinan Series.
(21344)

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The analgesic and antitussive activities of the morphinan series have been studied by Benson *et al.*(1), while the cardiovascular effects were investigated by Randall *et al.*(2). It was felt that further insight into the action of these drugs would be gained by making simultaneous observations of their central and peripheral effects on the same animal. The present report describes the effects of 5 members of the morphinan series on the electroencephalogram (EEG), electrocardiogram (EKG) and blood pressure (BP) of the dog.

Methods. The technic is based on that described in previous papers(3,4). Dogs were prepared under thiopental anesthesia and then immobilized with decamethonium (C_{10}). Artificial respiration, applied through a tracheal cannula, was maintained throughout the experiment. The EEG was recorded from steel needles inserted in holes drilled through the skull; the EKG (lead II) was recorded from needles in the skin, while the BP was measured through a hypodermic needle in the right femoral artery. All drugs were injected into the left femoral vein. In experiments on direct action of drugs, only a single drug was given to each dog; the injection was made four hours after the last dose of thiopental. In experiments on drug antagonism, a second drug was injected 2 minutes after the initial drug. The following drugs were studied: levorphan tartrate (*levo*-3-hydroxy-N-methylmorphinan tartrate); dextrorphan tartrate (*dextro*-3-hydroxy - N - methylmorphinan tar-

TABLE I. Direct Action of Drugs. All drugs tested at 1 mg/kg, I.V. Each figure is average of 3 exp.

	Avg % change				
Drug	Blood pressure	Heart rate	EEG frequency	EEG amplitude	Duration of effect, min.
Levorphan tartrate	–23	–68	–64	+461	120-180
Dextrorphan tartrate	–76	–46	–73	– 33	30- 90
Levomethorphan hydrobromide	– 3	–32	–49	+226	30- 90
Dextromethorphan hydrobromide	–33	–21	–70	+359	30- 60
Levallorphan tartrate	+2	–33	–29	+238	10- 30

trate); levomethorphan hydrobromide (*levo*-3-methoxy-N-methylmorphinan hydrobromide); dextromethorphan hydrobromide (*dextro*-3-methoxy-N-methylmorphinan hydrobromide); levallorphan tartrate (*levo*-3-hydroxy-N-allylmorphinan tartrate).

Results. Preliminary experiments with levorphan indicated that the maximum EEG effects occurred at 1 mg/kg, I.V. The remainder of the experiments were therefore performed at this dose level. Experiments on direct action of these compounds are summarized in Table I. (Fig. 1 and 2.) The following points deserve comment:

Blood pressure. The greatest fall in BP in this series was produced by dextrorphan, followed by dextromethorphan and levorphan. Levomethorphan and levallorphan had no appreciable BP effect at the dose level tested. The depressor effect of morphine was attributed to a peripheral action of the drug by Schmidt and Livingston(5), while a similar finding regarding compounds of the morphinan series was made by Randall *et al.*(2). Hence the hypotensive effects described in the present report are probably caused by a peripheral action of the drugs.

Heart Rate. All the drugs produced a slowing of the heart; the effect was greatest with levorphan, followed by dextrorphan. The bradycardia induced by morphine in the dog has been attributed to a central action of the drug(5,6). This makes it probable that the bradycardia induced by the morphinan series is likewise due to a central action.

EEG Changes. All the drugs except dextrorphan produced large, slow waves in the EEG. Similar waves have been observed in dogs under barbiturate anesthesia or during natural sleep(3), and probably are an indication of central depression. Since changes in EEG amplitude can be measured more ac-

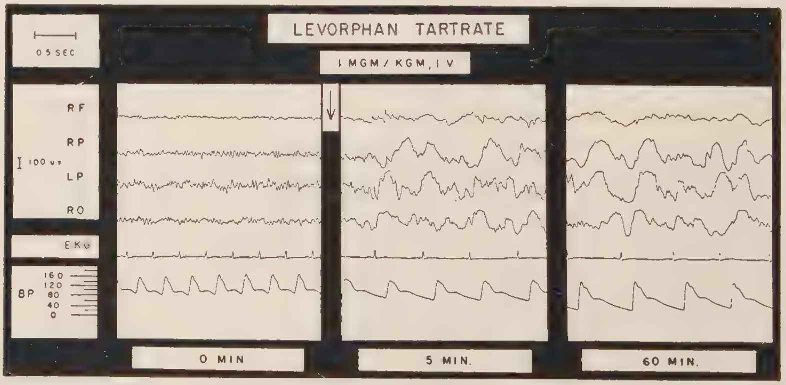

FIG. 1. Effects of levorphan tartrate, 1 mg/kg, I.V. in dog. The first 4 lines in each record are the EEG, right frontal, right parietal, left parietal and right occipital leads. The fifth line is the EKG, Lead II, and the bottom line is the blood pressure (BP). Paper speed, 6 cm/sec. Mar. 9, 1954. Dog No. 410, male, 9.7 kg. Record A—Control; BP 165 mm Hg; heart rate (HR) 180 per min.; dominant EEG pattern, 10/sec., 50 μV. Record B—5 min. after drug; BP 160 mm; HR 108/min.; EEG 2/sec., 320 μV. Record C—60 min. after drug; BP 155 mm; HR 96/min.; EEG 2/sec., 310 μV.

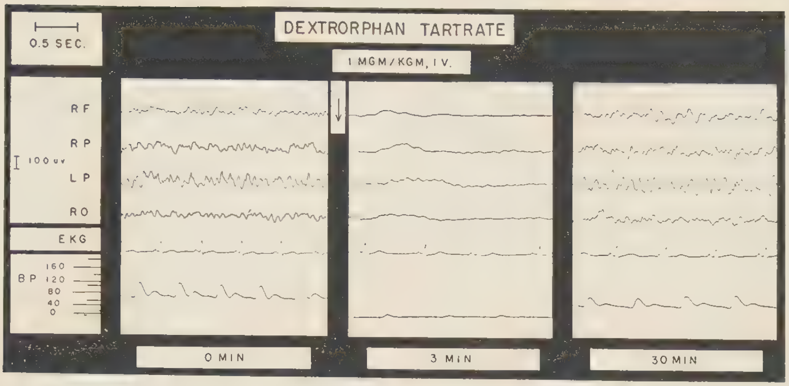

FIG. 2. Arrangement as in Fig. 1. Effects of dextrorphan tartrate, 1 mg/kg, I.V. April 12, 1954. Dog No. 422, male, 7 kg. Record A—Control; BP 120 mm; HR 120/min.; EEG 6/sec., 100 μV. Record B—3 min. after drug; BP 20 mm; HR 84/min.; EEG flat. Record C—30 min. after drug; BP 90 mm; HR 84/min.; EEG 8/sec., 130 μV.

curately than those in frequency, the increase in amplitude may be used as a measure of this central effect. The largest increase was produced by levorphan, followed by dextromethorphan, levallorphan and levomethorphan. The EEG effects of dextrorphan differed from those of the other drugs studied. It was the only compound producing an average decrease in amplitude. In 2 out of 3 experiments, the EEG was completely flattened (Fig. 2). This flattening of the EEG resembles that previously obtained with the hypotensive drug, Arfonad camphorsulfonate(7). This effect was attributed to the sharp fall in BP following rapid injection of Arfonad. Hence the flattening of the EEG obtained with dextrorphan is probably caused by the fall in BP produced by this drug, rather than by any direct central action.

Antagonism to levorphan. In a second series of experiments, we investigated the antagonism to levorphan of the remaining members of the series (Table II). Levallorphan was the only drug which consistently reversed the effects of levorphan. Both central and peripheral effects were antagonized. Levallorphan was most effective when injected into an animal already depressed by levorphan, since no antagonism was shown when levallorphan was injected before levorphan, and only partial antagonism was shown when the drugs were injected simultaneously. Antagonism by levallorphan of the analgesic, respiratory and circulatory effects of levorphan has been described by previous workers(2,8,9). The present paper extends these observations to the EEG effects of Levorphan.

TABLE II. Antagonism to Levorphan. All drugs tested at 1 mg/kg, I.V. Second injection given 2 min. after first injection. 8 dogs.

1st inj.	2nd inj.	Antagonism
levorphan	dextrorphan	None
”	levomethorphan	”
”	dextromethorphan	”
”	levallorphan	Complete
levallorphan	levorphan	None
‘‘pre-mixed’’ levorphan + levallorphan	—	Partial

Discussion. A striking finding in our work is the difference in activity between levorphan and dextrorphan (compare Fig. 1 and 2). Levorphan has strong EEG effects and mild hypotensive action. These relations are reversed in dextrorphan, which produces strong cardiovascular effects with little or no direct central action. A similar relationship has been described between morphine and pseudomorphine (Schmidt and Livingston)(10). These workers found that "pseudomorphine retains in exaggerated form the vasodilator effects of morphine, but it has practically none of the direct actions of morphine on the central nervous system." This similarity in action of pseudomorphine and dextrorphan presents an interesting problem.

Summary. The effects of 5 members of the morphinan series have been studied on the electroencephalogram, electrocardiogram and blood pressure of the dog. Levorphan produced maximum central effects with mild cardiovascular depression. Dextrorphan produced severe cardiovascular depression with little or no direct central effect. Levomethorphan and dextromethorphan had mild central and peripheral actions. Levallorphan, which had little action by itself, antagonized both the central and peripheral effects of levorphan.

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Alloxan Diabetes in Different Strains of Mice.* (21345)

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Alloxan diabetes in mice has been obtained by Kirschbaum and Bell(1) by subcutaneous injection of 200 mg/kg, and Lazarow(2) reported that intravenous diabetogenic dose was between 50 and 75 mg/kg of body weight, without mentioning the strain of mice.

Since the diabetogenic action of alloxan in mice could depend upon the strain used, and since in this laboratory some genetically homogeneous stocks of mice are maintained by close inbreeding, an attempt has been made to find out whether a difference in susceptibility to alloxan could be found by testing different strains of mice. Oral glucose tolerance tests were performed in normal as well as in diabetic animals and early blood sugar changes after alloxan have been determined also in normal mice.

Method. Male mice of the C_3H, A, C and D_8 strains were used. Alloxan monohydrate was injected intravenously into the tail vein after warming the mouse to facilitate injection. A 22 gauge hypodermic needle was used. Alloxan was injected as a freshly prepared 0.5 to 1% solution in saline. Blood samples (0.05 cc) were taken 48 hours to 1 week later by cutting the tip of the tail and the sugar determined by the Somogy-Nelson micromethod (4). Since the normal blood sugar level after 3 hours fasting ranged between 100 and 130 mg/ml of blood in most of the strains used, only those mice showing a blood sugar greater than 150 mg/100 ml after 3 hours fasting were considered as diabetic.

The *results* are shown in Table I. The injection of 40 mg/kg produced diabetes in 1 out of 9 and in 1 out of 10 mice of the strains A and D_8, respectively. Sixty mg/kg produced diabetes in 5 out of 11 mice of the C stock, in 5 out of 10 of the A, and in 9 out of 10 of the D_8, whereas the same amount failed to produce diabetes in mice of the C_3H stock. Eighty mg/kg produced diabetes in 6 out of 10 of the C_3H mice, while all animals of the C, A and D_8 stocks became diabetic. The average blood sugar level of diabetic animals was higher in mice of the D_8, A and C strains than in the C_3H.

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* Assisted by an institutional grant from the American Cancer Society.

TABLE I. Sensitiveness to Alloxan Injected Intravenously in Male Mice of Different Strains.

	40 mg/kg intrav.			60 mg/kg intrav.			80 mg/kg intrav.		
Strain	No. of mice	Diabetic, %	Blood sugar (mean)*	No. of mice	Diabetic, %	Blood sugar (mean)*	No. of mice	Diabetic, %	Blood sugar (mean)*
C_3H	10	0	133	18	0	127	10	60	179†
C	10	0	120	11	44	207†	8	100	335
A	9	11	152†	10	50	179†	10	100	391
D_8	10	10	152†	10	90	240†	10	100	444

* mg/100 ml. † Blood sugar of diabetic.

Glucose tolerance test in diabetic and non-diabetic mice. In a group of diabetic and normal C male mice, glucose tolerance test was performed. After 15 hours fasting, both groups were fed by stomach tube with glucose (3 g/kg) and blood samples taken every successive 30 minutes throughout a 2½ hour period. The experiment was necessarily carried out in different groups of mice and the results plotted in a curve (Fig. 1) in which each point represents the mean sugar level of 5 animals. Normal mice exhibited a rise in blood sugar at 30 minutes, returning to the normal level 2½ hours later, whereas the diabetic showed a much greater increase in blood sugar at 30 minutes, remaining hyperglycemic after 2½ hours.

Response of blood sugar to alloxan. Since no reports have been noted on the response of blood sugar to alloxan in the mouse, experiments were undertaken using C male mice injected with a diabetogenic dose of alloxan (80 mg/kg) intravenously. The results showed that alloxan injection was followed by typical triphasic blood sugar changes characteristic of most experimental animals.

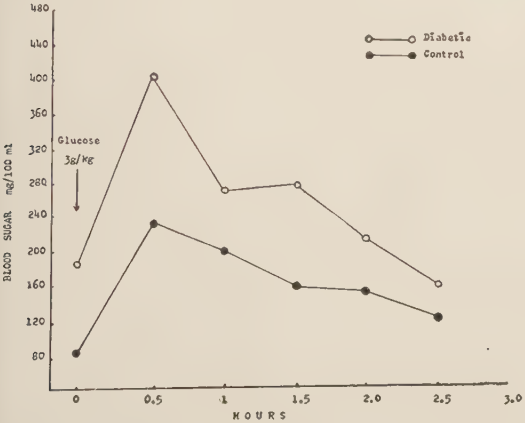

FIG. 1. Oral glucose tolerance test (3 g/kg) in diabetic and non-diabetic C male mice.

Discussion. The results demonstrate that a difference in susceptibility to alloxan diabetes in mice can be found, depending upon the strain used. In fact, mice of the C_3H stock showed a greater resistance than those of the C, A and D_8 stocks, the latter being particularly susceptible. The difference between the C_3H and the D_8 groups when 60 mg/kg was injected is statistically significant at 1% level, and between the C_3H and either the A or the C is significant at the 5% level, determined by the tables for use in the 4-fold contingency test of Mainland and Murray(3).

The glucose tolerance test in alloxan treated mice showed typical changes in blood sugar characteristic of the diabetic condition. Also changes in blood sugar after injection of alloxan were quantitatively similar to those described in most experimental animals.

Summary. 1. Male mice of the C_3H, C, A and D_8 strains are susceptible to the diabetogenic action of alloxan. A difference in susceptibility depended upon the strain, the D_8 was the most susceptible and the C_3H, the most resistant, to a given amount of alloxan injected. 2. Glucose tolerance tests in diabetic as well as in non-diabetic mice are presented. The blood sugar changes after intravenous injection of alloxan showed the triphasic type curve described in most experimental animals.

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Determination of Pyruvate-activated Glutaminase II Activity of Liver Tissue.* (21346)

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Two separate glutaminase enzymes have been demonstrated in rat liver tissue—glutaminase I (phosphate-activated) and glutaminase II (pyruvate-activated). Glutaminase I occurs in numerous animal tissues both normal and neoplastic(1,2), whereas glutaminase II is apparently restricted to liver tissue and liver tumors(1,3). Errera(4) successfully separated these two enzymes from an aqueous homogenate of rat liver by high speed centrifugation (glutaminase I in sediment; glutaminase II in supernatant) and found the optimal pH for activity of purified glutaminase II to be 7.7. Using an aqueous extract of rat liver, Greenstein and Price(5) found the optimal pH to be 7.2 and further found that α-ketoisocaproate activated glutaminase in a manner analogous to pyruvate; other keto acids were reported to be without effect on the deamidation of glutamine(6).

This communication describes a method for the determination of pyruvate-activated glutaminase II activity which combines speed, simplicity and accuracy of results, suitable for routine determinations. The method of Greenstein and Price(5) entails a relatively long incubation period of 4 hours followed by aeration of ammonia into sulfuric acid traps prior to nesslerization. It was not stated if the rate of reaction was constant up to 4 hours of incubation. In the present method, the reactants were incubated for 2 hours at which time the rate of reaction was constant with optimal concentrations of glutamine and pyruvate and at the optimal pH. Ammonia was determined by direct nesslerization of the reactant mixture following precipitation of proteins. Liver glutaminase II activities have been determined for 6 animal species including humans.

Experimental. Adult male albino rats of the Wistar strain were fed standard fox chow *ad libitum* with drinking water freely available. Rats were fasted for 18 to 24 hours, stunned and decapitated. Livers were quickly removed, a portion rinsed in cold glass-distilled water, blotted, accurately weighed, and homogenized in cold glass-distilled water with the aid of a Potter homogenizing tube immersed in ice-water. The homogenate was lightly centrifuged at about 1500 RPM for 5 minutes, the sediment of tissue debris and insoluble matter discarded and the supernatant aqueous extract used. This extract was stored in an ice-water bath and assayed within 30 minutes of preparation. Each reaction tube (in duplicate) contained 1 ml liver extract, 1 ml L-glutamine, 1 ml sodium pyruvate (adjusted to pH 7.7) and 1 ml 0.01M veronal buffer (pH 7.7). A blank containing 1 ml liver extract, 1 ml L-glutamine, 1 ml veronal buffer and 1 ml glass-distilled water was prepared for each sample.

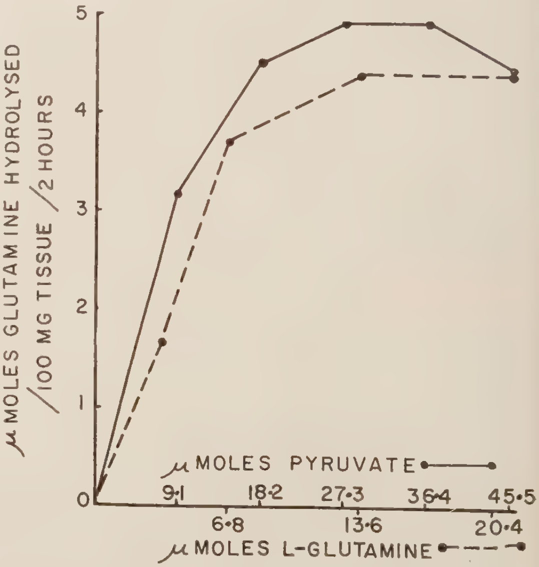

FIG. 1. Optimal L-glutamine and sodium pyruvate concentrations. Each tube contained 40 mg liver tissue, 0.01M veronal buffer (pH 7.7), sodium pyruvate (pH 7.7) and L-glutamine. Total vol, 4 ml. Incubated 2 hr at 37°C. Effect of pyruvate tested with 20.4 µmoles L-glutamine. Effect of L-glutamine tested with 31.8 µmoles sodium pyruvate.

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* This work was supported by a grant from the National Research Council of Canada for which the author expresses his appreciation.

This blank corrected the following possible errors, % of total ammonia contributed by each in a typical rat liver assay (40 mg tissue) being given in parenthesis: preformed ammonia and non-enzymatic hydrolysis of glutamine (12.7); "apparent activity" in the absence of added pyruvate (38.2). Archibald(7) has discussed the non-enzymatic hydrolysis of glutamine on addition of Nessler reagent. Following incubation with constant agitation in a 37°C water bath, 2 ml 0.7 N sulfuric acid was added to each tube followed by 2 ml 7% sodium tungstate and the contents of each tube were filtered to remove precipitated proteins. Blanks and the ammonium sulfate solutions used in preparing a standard curve were similarly filtered since filter paper may contaminate solutions with small amounts of ammonia. Four ml of filtrate was added to 4 ml of Archibald's persulfate-gluconate stabilizer(7) diluted 2:1 with water and 1 ml of Vanselow's Nessler reagent(8) was rapidly added to the solutions with a blow-out pipette. Five minutes after the addition of Nessler reagent, developed color was read in a Coleman Junior spectrophotometer at a wavelength of 450 mu.

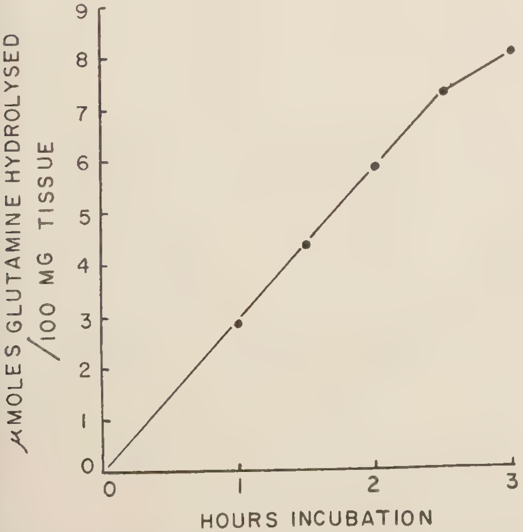

FIG. 2. Time course of reaction. Each tube contained 40 mg liver tissue, 20.4 μmoles L-glutamine, 31.8 μmoles sodium pyruvate (pH 7.7), 0.01M veronal buffer (pH 7.7). Total vol, 4 ml. Incubated at 37°C.

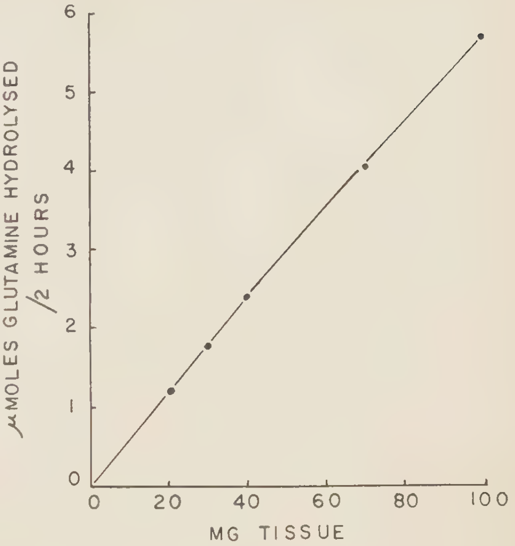

FIG. 3. Tissue concentration and enzyme activity. Each tube contained 20.4 μmoles L-glutamine, 31.8 μmoles sodium pyruvate (pH 7.7), 0.01M veronal buffer (pH 7.7). Total vol, 4 ml. Incubated 2 hr at 37°C.

Results. Effect of pH on enzyme activity was tested from pH 6.0 to 9.5 and the optimal pH appeared to be 7.7 as found by Errera for the isolated enzyme(4). The L-glutamine and sodium pyruvate used in these studies were obtained from Nutritional Biochemicals Corporation. The data of Fig. 1 resulting from assays of 2 different rats demonstrate the optimal concentration of L-glutamine to be 20.4 μmoles and that of pyruvate to be 31.8 μmoles. These concentrations were chosen for subsequent studies. Two hours incubation at 37°C has been chosen for the present studies because at this time the rate of reaction is constant (Fig. 2) and the amount of formed ammonia is easily determined. The data (Fig. 2) further indicate that preincubation of tissue with pyruvate for activation is not necessary. The data of Fig. 3 demonstrate a linear relation between enzyme activity and tissue concentration up to 40 mg tissue with a very gradual diminution in rate of reaction above this concentration.

Using a 4% aqueous extract of liver (40 mg tissue), 31.8 μmoles sodium pyruvate (pH 7.7) and veronal buffer (pH 7.7), ammonia formation was measured with 20.4 μmoles of L-glutamine or L-asparagine (Eastman) or L-glutamic acid (Nutritional Biochemicals Corporation) as substrate. The reactants were incubated for 2 hours at 37°C. The μmoles substrate hydrolysed were as fol-

TABLE I. Liver Pyruvate-Activated Glutaminase II Activities in Adult Male Animals.

	No. of animals	Glutaminase II activity* (mean ± stand. dev.)
Rats	5	5.01 ± .65
Mice	5	4.77 ± 1.10
Rabbits	5	3.29 ± 1.06
Guinea pigs	8	1.42 ± .10
Monkeys	10	None detected

* Expressed as μmoles glutamine hydrolysed by 100 mg tissue in 2 hr. All values corrected for hydrolysis of glutamine in absence of added pyruvate.

lows: 5.45 for L-glutamine, 9.34 for L-asparagine and 0 for L-glutamic acid per 100 mg tissue in 2 hours. Since glutamic acid did not yield free ammonia under these conditions, it is safe to assume that ammonia arising from glutamine represents hydrolysis of the amide group only. The marked deamidation of asparagine would be expected since rat liver extracts contain an active α-keto acid-activated asparaginase(5).

Liver glutaminase II activities in normal animals. Although pyruvate-activated glutaminase activity has been observed in the livers, though not other tissues, of rats and mice, Errera and Greenstein(1) were unable to find appreciable activation of guinea pig or rabbit liver glutaminase with either added phosphate or pyruvate. Since their experimental conditions differed from those of the present method, it was felt advisable to reinvestigate glutaminase II activities in livers from guinea pigs and rabbits. The Rhesus monkey was also investigated and for comparative purposes, enzyme activity was measured in liver tissue of albino rats of the Wistar strain and albino mice of the Connaught strain. With each tissue sample, a blank containing glutamine, tissue and buffer (pH 7.7) was run to correct the possible errors already mentioned. Forty mg liver tissue was used for rats and mice and 100 mg liver tissue for guinea pigs, rabbits and monkeys. The results of enzyme assays are shown in Table I.

The values obtained for rats and mice are about three times those found by Errera and Greenstein for rats(6) when calculated on the same basis. It should be noted that direct comparison of the present values with those of Errera and Greenstein is not valid since experimental conditions differ. Whereas the values reported here were derived with 2 hours' incubation at a constant rate of reaction and express rate of activity, Errera and Greenstein (6) employed an incubation period of 4 hours. The time course of reaction presented here suggests that after 2½ hours of incubation, the rate of reaction diminishes. Errera has shown a similar time course with the purified enzyme (3). The pyruvate catalyzed activities found in rabbit livers although lower than rat or mouse liver were not inappreciable as suggested by the earlier finding of Errera and Greenstein(1). Indeed, the mean value of rabbit liver was two-thirds that of rat liver. Pyruvate-catalyzed deamidation of glutamine in guinea pig liver was very slight in agreement with the earlier finding(1). No pyruvate-activated glutaminase II activity was detected in any of the monkey livers studied, suggesting (a) absence of this enzyme or (b) the optimal conditions for estimating this enzyme in monkey liver are markedly different than in the other species studied. The first explanation seems more plausible since not even slight activity was noted in any of the 10 livers.

Glutaminase II in human liver tissue. The findings of glutaminase II activity in the livers of some animal species but no apparent activity in monkey liver suggested an investigation of human liver. Human liver tissue was obtained at autopsy from 5 subjects, the causes of death being acute leukemia, hypernephroma, meningioma, ruptured auricular appendage and suffocation.† Time elapsing between death and enzyme assay ranged from 5 to 24 hours. Glutaminase II determinations were done immediately using 100 and 200 mg tissue and accompanied by determinations of liver arginase activity(9) on 4 of the samples as a control measure. Pyruvate-activated glutaminase II activity could not be detected in any of the samples although arginase activity was found in all of the 4 livers tested, activities ranging from 10 to 20 μmoles urea formed by 100 mg tissue in 1 hour. In a second control experiment, glutaminase II and arginase activities were determined in livers of 5 mice immediately after death and of 5 mice 6 hours after death. Appreciable activity of both glutaminase II (52%) and arginase (57%) was found 6 hours after death. It should be noted that 2 of the human liver samples were assayed within 6 hours of death yet no glutaminase II activity was detected although arginase activity was found.

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† The author is indebted to Dr. H. J. Barrie, Department of Pathology, University of Toronto for supplying human liver samples.

Discussion. Meister *et al.*(10-13) have reported a rat liver glutamine- (or asparagine-) transamidase-deamidase system. In this system the formed ammonia arises from the amide group of glutamine or asparagine and deamidation does not precede transamination (12). Unlike other transamination systems, glutamine transamination was unaffected by vitamin B_6 deficiency in the rat(13). These workers concluded that "α-keto acid-catalyzed deamidation of glutamine and transamination are associated"(12).

Summary. A simple and rapid method is described for estimating pyruvate-activated glutaminase II activity of liver tissue. Glutaminase II activity was found in livers of rats, mice and rabbits but was absent in livers of monkeys and was low in livers of guinea pigs. Using human liver tissue taken at autopsy, glutaminase II activity could not be detected although arginase activity was found in these livers.

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Effect of Adrenocortical Hormones on Presence of C-reactive Protein in Blood.* (21347)

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(Introduced by William S. Tillett.)

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The demonstration of C-reactive protein (CRP) in human blood has been shown to be a highly sensitive and reliable test for the presence of low grade inflammation in patients with acute rheumatic fever and other diseases (1-4). C-reactive protein disappears from the blood during effective treatment with cortisone, ACTH or salicylates. This has been considered to indicate suppression of the inflammatory process(4).

It is also possible, however, that these antirheumatic agents may exert a direct, catabolic effect upon the metabolism of CRP. If this were so the usefulness of the test for CRP in blood as a guide to the effective suppression of rheumatic inflammation would be limited. This study attempts to determine whether the administration of ACTH, cortisone or salicylates alters the promptness, intensity and duration of the appearance of CRP in human

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† Fellow in Rheumatic Fever, N. Y. State Department of Health.

TABLE I. Appearance of C-Reactive Protein in Blood of 9 Human Beings following Administration of Typhoid Vaccine Intravenously before and during Treatment with Cortisone or Salicylates.

Patient	Typhoid bacilli (millions)	Treatment†	Hr after injection of typhoid vaccine						
			0	12	24	48	96	120	144
J.V.	37.5	0	0	+	+	+	0	0	0
	37.5	A*	0	+	2+	+	±	0	0
N.R.	37.5	0	0	+	+	+	0	0	0
	37.5	A*	0	+	2+	+	+	0	0
R.D.	75	0	0	2+	4+	3+	2+	±	0
	125	C*	0	2+	3+	2+	0	0	0
J.D.	100	0	0	—	2+	2+	+	+	0
	50	C*	0	—	2+	+	+	—	0
R.Z.	100	C*	0	2+	3+	3+	0	+	0
	50	0	0	2+	2+	+	0	0	0
B.S.	75	0	0	2+	2+	3+	2+	+	0
	125	C*	0	2+	3+	3+	0	0	0
W.G.	50	0	0	+	3+	3+	3+	2+	0
	100	C*	0	3+	4+	4+	0	0	0
P.O.	37.5	0	0	—	+	+	±	0	0
J.C.	70	ACTH	0	+	2+	+	0	0	0

* Treatment started 4 days before typhoid vaccine administered and continued for 7 days afterward.

† A = Aspirin; C = Cortisone.

+, Positive test for CRP. 0, Negative. —, Not done.

blood following the application of an artificial stimulus. In addition, studies have been made of the effect of cortisone and corticotropin upon the behavior of the analogous Cx-reactive protein of rabbits(5) infected with pneumococci.

Materials and methods. Observations in human subjects: Typhoid vaccine‡ was administered in varying dilutions as a single intravenous injection to 9 patients, ranging in age from 9 to 15 years, who had recovered completely from a recent attack of rheumatic fever and who had no clinical evidence of cardiac involvement. Blood samples were obtained before administration of vaccine and at 6, 12, 24, 48, 96, 120 and 148 hours thereafter. Each sample was tested for the presence of C-reactive protein by a capillary precipitin reaction employing a specific rabbit anti-human C-reactive protein antiserum(3).§ Positive reactions were graded as 1+ to 6+ based on the volume of precipitate formed. Thus, a value of 6 plus was assigned to a precipitate which filled the capillary tube completely and a value of one plus to a precipitate which filled one-sixth of the total column of fluid in the capillary tube. All tests were read after the mixture of serum and specific rabbit antiserum had been incubated for 2 hours at 37°C and allowed to stand at 4°C overnight (approximately 18 hours). Determinations of erythrocyte sedimentation rate were made by the Wintrobe method and the values were corrected for variations in hematocrit according to standard tables. Rectal temperature was recorded every half hour during the febrile reaction and every 4 hours thereafter. The procedure was repeated in the same patients during the administration of either aspirin, cortisone or ACTH. Aspirin was administered by mouth in doses of one grain per pound of body weight per day. Cortisone was administered in daily doses of 300 to 400 mg in most patients. Either medication

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‡ The vaccine was obtained from N. Y. City Department of Health and contained the following: *S. typhosa* 1,000 million organisms/ml; *S. paratyphi* A, 250 million organisms/ml and *S. paratyphi* B 250 million organisms/ml.

§ The antiserum was kindly supplied by Drs. Maclyn McCarty and Harrison Wood of the Hospital of the Rockefeller Institute.

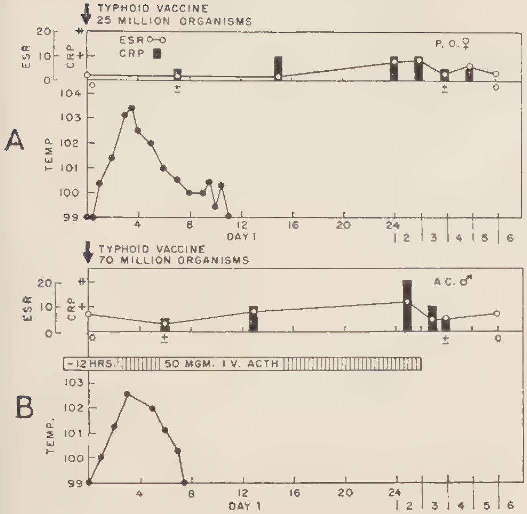

FIG. 1. Appearance of C-reactive protein in human blood following intravenous injection of typhoid vaccine before (A) and during (B) administration of ACTH intravenously.

was given for a period of several days prior to the administration of typhoid vaccine and treatment was continued for 7 days thereafter. ACTH was administered by continuous intravenous drip to one patient in a dose of 50 mg in 48 hours. Treatment was initiated 12 hours before the injection of typhoid vaccine. *Experiments with rabbits.* New Zealand or hare brown rabbits weighing about 3.5 kg were infected by intradermal injection of 0.2 ml of an undiluted 18 hour culture of type 1 pneumococci. One group of animals was treated with a single daily intramuscular injection of 10 mg of cortisone acetate. A second group of rabbits received 10 mg per day of ACTH in 4 equal injections at 6 hour intervals. A third untreated group served as controls. The animals were bled from the ear every 6 hours for the first 24 hours and daily thereafter for 4 days. Each sample of blood thus obtained was tested for the presence of Cx-reactive protein by a precipitation reaction employing a solution of pneumococcal Cx-polysaccharide(5). In some instances a capillary precipitin test was performed employing a specific rooster anti-rabbit Cx-reactive protein antiserum.

Results. Effect of antirheumatic agents upon CRP response to intravenously administered typhoid vaccine. Following a single intravenous injection of typhoid vaccine CRP usually appeared in the blood within 6 to 12 hours, reached maximum levels within 24 to 48 hours, and persisted for 4 to 5 days (Table I). The minimum dose required to produce this response was approximately 25 million bacilli (Fig. 1A).

The administration of cortisone, aspirin or ACTH failed to alter significantly the promptness, intensity or duration of the CRP response in any of the patients studied (Table I). Some experiments were designed to detect minor alterations in the CRP response in the treated patients by injecting close to a "threshold" stimulus of typhoid vaccine. No differences were observed in the CRP response of these patients during cortisone or aspirin treatment as compared with the preliminary "control" experiment (Fig. 2 A, B).

In other experiments larger doses of typhoid vaccine were employed to determine whether roughly quantitative differences in the CRP response could be detected during the administration of cortisone. No consistent suppression of the CRP response was observed even when the dose of typhoid vaccine administered during the period of cortisone treatment was half as great as that given in the preliminary "control" experiment. Similarly, the administration of ACTH intravenously to one patient did not appear to alter the CRP response to the typhoid vaccine stimulus (Fig. 1B).

Effect of cortisone and ACTH on the Cx-reactive protein response in rabbits. Cx-reactive protein appeared in the blood of rabbits within 12 to 18 hours after the intradermal injection of live pneumococci and persisted in all surviving animals during the 4 day period of the experiment. The administration of cortisone or ACTH to rabbits did not appear to influence significantly the Cx-reactive protein response to the experimental infection. Cx-reactive protein appeared in the blood of fewer rabbits in the cortisone treated group at 18 hours. However, by 24 hours more animals in this group had detectable amounts of the abnormal pro-

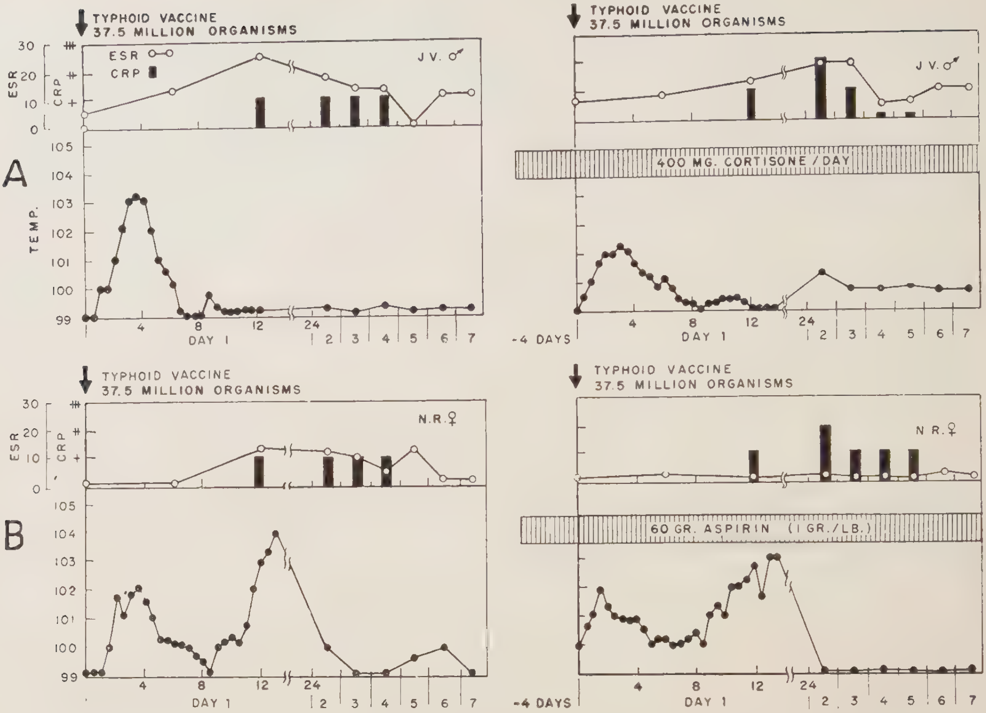

FIG. 2. Appearance of C-reactive protein in human blood following intravenous injection of typhoid vaccine before and during administration of cortisone (A) and aspirin (B).

tein in their blood. After 24 hours, when septicemia had ensued, virtually all surviving animals in both the treated and untreated groups had detectable amounts of the abnormal protein in their blood (Table II). The average survival time of the hormone treated rabbits was shorter than that of the control group. Two of 13 untreated rabbits survived the infection. There were no survivors in the hormone treated group.

Discussion. In rabbits the appearance in blood of Cx-reactive protein has been described during intravenous immunization with protein antigens(6-7). In the present study the pattern of this response in human beings was found to be quite consistent in the same subject and was not altered significantly by the administration of cortisone, ACTH, or aspirin. This was true even in those patients in whom the expected rise in erythrocyte sedimentation rate did not recur during the period of treatment (Fig. 2B).

TABLE II. Incidence and Time of Appearance after Infection of Cx-Reactive Protein.

No. of rabbits	Therapy, mg/day	Hr					
		0	12	18	24	48	72
15	Cortisone 10	*0/15	1/7	3/7	12/14	8/8	3/3
14	ACTH 10	1/14	2/7	5/7	8/14	7/8	3/3
13	0	0/13	2/7	6/7	8/13	11/11	6/6

* No. of rabbits with positive test for Cx-reactive protein divided by No. of survivors tested.

With the doses of the antirheumatic agents employed it was not possible to suppress completely the febrile response which followed injection of typhoid vaccine. Because of this it might be argued that either inadequate amounts of cortisone, ACTH or aspirin were given or that too strong a stimulus to CRP formation had been applied. Either argument would not appear to be relevant, however, to the interpretation of the behavior of the CRP in the blood of patients with acute rheumatic fever when the usual therapeutic doses of aspirin, cortisone or ACTH are administered. Under these conditions CRP rapidly disappears from the blood coincident with the subsidence of clinical signs of rheumatic inflammation(4). Conversely, administration of cortisone in doses as high as 400 mg per day may not cause disappearance of CRP in the patient's blood if clinical signs of rheumatic inflammation persist(4). CRP appears to remain in the blood as long as an effective inflammatory stimulus is present and disappears from the blood only when inflammation is suppressed or subsides spontaneously.

The situation appears to be analogous when a pyrogenic reaction is elicited by the intravenous administration of a foreign protein. In this circumstance the stimulus to the production of CRP cannot be blocked by the adrenalcortical hormones or by salicylates. C-reactive protein appears promptly in the blood and seems to remain until the stimulus to its production is dissipated, even in the presence of doses of cortisone which would ordinarily suppress most signs of rheumatic inflammation. Cortisone therefore does not appear to block directly the formation, or to accelerate the degradation, of C-reactive protein.

In rabbits, the response of the analogous C-reactive protein appears to follow the progress of experimental infection whether or not the adrenal cortical hormones have been administered. These results are in agreement with similar studies in rabbits by Good(8).

Summary and conclusions. 1. The C-reactive protein response in human blood following intravenous injections of typhoid vaccine is described. No significant alteration in the promptness, intensity or duration of this response was noted during the administration of cortisone, ACTH or salicylates. 2. In rabbits the response of the analogous Cx-reactive protein to experimental pneumococcal infection is similarly not modified by the adrenal cortical hormones. 3. The experimental results support the clinical impression that the disappearance of C-reactive protein from human blood during antirheumatic therapy with adrenalcortical hormones or with salicylates is secondary to suppression of the inflammatory process rather than a primary effect of these agents upon the metabolism of the C-reactive protein.

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Effect of Two Isoniazid Derivatives and Pas Hydrazide on Isoniazid Susceptible and Resistant Tubercle Bacilli.* (21348)

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Since the discovery of the antituberculous activity of isoniazid (INH), many related chemical compounds have been studied in the hope of finding even more powerful inhibitors of the tubercle bacillus, or drugs with even less toxicity for man. Another qualification to be sought is undiminished action upon isoniazid resistant strains, for the emergence of such strains in patients, first demonstrated in 1952 (1), has become one of the major factors to be reckoned with in the clinical use of this drug.

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* This study was aided by grants from the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service.

TABLE I. *In Vitro* Tuberculostatic Effect of N-Isonicotinoyl-N′-(salicylidene) Hydrazine (INSH), N-Isonicotinoyl-N′-(2,2-dimethyl-3-hydroxypropylidene) Hydrazine (IHPH), and PAS Hydrazide (PASH) in 3 Different Liquid Media after 14 Days Incubation. Minimal inhibitory concentrations in μg/ml.

		Medium		
Drug	Test strain	Tween-albumin	Proskauer & Beck	Proskauer & Beck with 10% horse serum
INSH	H37Rv	.06– 0.1[*]	.06–.1	.3
	H37Rv resis. to INH[†]	10 – 25		
	4827[‡]	2.5 – 5		
	F.N.[‡]	2.5 – 5		
	E.R.[§]	.5 – 1		
IHPH	H37Rv	.03– .06	.03–.06	.6 –1.2
	H37Rv resis. to INH[†]	25		
PASH	H37Rv	.6 – 1.25	.15–.3	1.25–2.5
	H37Rv resis. to INH[†]	.6 – 2.5		
	H37Rv resis. to PAS[†]	50 –100		

[*] Two figures separated by dash indicate spread of results on repeated tests.

[†] Strain made highly resistant to INH or PAS by repeated passages in drug-containing liquid medium.

[‡] Strain isolated from patient treated with INH; resistant to 1, partially resistant to 5, and sensitive to 10 μg of INH/ml of solid medium.

[§] Strain isolated from patient treated with INH; resistant to 0.1, sensitive to 1 μg of INH/ml of solid medium.

This report concerns the *in vitro* and *in vivo* antituberculous effect against INH-sensitive and -resistant tubercle bacilli of 3 chemical compounds related to INH, namely, the hydrazide of p-aminosalicylic acid (PASH), N - isonicotinoyl - N′-(salicylidene) hydrazine (INSH), and N-isonicotinoyl-N′-(2, 2-dimethyl-3-hydroxy-propylidene) hydrazine (IHPH). The assessment of the value of chemotherapy in guinea pigs infected with INH-resistant tubercle bacilli was delayed for over a year by the unexpected finding of loss of pathogenicity for these animals of strains made resistant *in vitro,* and of many resistant strains freshly isolated from patients(2-10). In the meantime, others (11-13) reported successful infection of mice with such strains. Eventually, however, several cultures exhibiting different degrees of INH resistance and retaining full virulence for guinea pigs were recovered from patients. Three of these human strains, together with one resistant bovine culture obtained from an experimental rabbit treated with INH, were used to infect guinea pigs.

In vitro experiments. The inhibitory activity of these compounds has been reported by several groups(14-19). The *in vitro* tuberculostatic effect was evaluated in 3 different liquid media. Stock solutions of INSH[†] and IHPH[†] were made in 95% ethyl alcohol in concentration of 2 mg/ml. The PASH[†] was dissolved in distilled water, 1 mg/ml. Serial 2-fold dilutions of each drug were made in 5 ml amounts of each medium in test tubes, and the inoculum of the test organism into each tube consisted of 0.1 ml of a 7- to 10-day culture in Tween-albumin medium. The results are shown in Table I. It may be seen that the 2 INH derivatives produced inhibition of growth in very low concentrations, similar to INH itself. Against the H37Rv strain which had been made highly resistant to INH *in vitro,* however, IHPH showed very little activity and INSH a greatly reduced activity. The increase in minimal inhibitory concentration of INSH for the 3 strains of intermediate INH resistance, #4827, FN, and ER, was about the same order of magnitude as that of INH for these same strains, namely 50x, 50x, and 10x, respectively. PASH showed about the same inhibitory effect as PAS, but retained its full activity against the H37Rv strain resistant to INH. It had very little effect, how-

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[†] Supplied by Nepera Chemical Co., Yonkers, N. Y., whose trade names for INSH and IHPH are Salizid and Pivalizid, respectively. Some PASH was also supplied by Panray Corp., New York City.

TABLE II. Isoniazid Resistance of Four Cultures Used to Infect Guinea Pigs on December 2, 1953.

Strain and date of isolation	Time of test	Growth* on solid medium slopes containing INH, μg/ml 0	.1	1	5	10	25	Approximate % of population resistant to INH, μg/ml .1	1	5	10
E.R.	Orig.	3+	2+	0	0	0	0				
4/16/53	Now†	4+	4+	50	0	0	0	70	.05	<.001	<.001
J.K.	Orig.	3½	3½	3+	2+	1+	1+				
4/16/53	Now	4+	3½	3+	0	0	0	60	40	<.01	<.01
9278-370	Orig.	4+	4+	4+	2+	50	0				
5/26/53	Now	4+	4+	4+	2+	0	0	100	100	1.0	<.001
F.N.	Orig.	4+	4+	4+	2½	0	0				
4/13/53	Now	4+	4+	4+	1+	0	0	100	100	5.0	<.005

* Final growth after 6-8 wk of incubation, graded according to following scale: No. of colonies if less than 51; 1+, 51 to 100 colonies; 2+, 100 to 300 colonies; 3+, innumerable colonies; 4+, confluent growth.

† Refers to tests performed at time of infection.

ever, against the PAS-resistant strain.

The ease with which the H37Rv strain could be made resistant to 2 of these compounds was investigated by serial transfer at 14- to 18-day intervals in Tween-albumin medium containing increased concentrations of the drugs. Only 8 transfers in INSH-containing medium were required to bring the resistance up to 25 μg/ml, after which the culture was also found to be highly resistant to INH, and to have lost its virulence for guinea pigs. Resistance to PASH developed a little more slowly, requiring about 10 transfers until the strain was resistant to 25 μg/ml, after which it was found to be highly resistant to PAS but still sensitive to INH. Resistance to the combination of INH plus INSH (in concentration ration of 1:2) developed even more slowly. In the 1st passage, the H37Rv strain was inhibited by a combination of 0.01 and 0.02 μg/ml of INH and INSH, respectively, and after 13 transfers was able to grow in 3.2 and 6.4 μg/ml of these drugs.

In vivo experiments. Guinea Pig. One hundred tuberculin negative 600-700 g guinea pigs were infected with 4 INH-resistant strains of tubercle bacilli, 25 with each strain. Three (ER, JK, and FN) were obtained from patients with pulmonary tuberculosis who had been treated with INH for at least 1 year, and all had produced progressive disease in guinea pigs when inoculated directly from the patient's sputum in April of 1953. The 4th strain (9278-370) was isolated from the lung of a rabbit which had died after 220 days of INH therapy after having been infected by inhalation of the virulent bovine strain, 4228-4. During the 6 to 7½ months between the time of original isolation and infection of the guinea pigs, these strains had been transferred twice on a solid egg-yolk, potato, glycerin medium without INH. The INH susceptibility of these strains at the time of original isolation and when they were used to infect the guinea pigs is shown in Table II. The former tests were carried out directly from the sputum concentrates onto tubes of solid medium containing various concentrations of INH, the latter on the same medium but from the 3rd generation subculture of the original strain. The approximate percentage of the population resistant to the various concentrations of INH was determined by plating out dilutions of the 3rd generation subcultures on INH-containing medium. Strains JK and 9278-370 evidently had lost the small percentage of individuals resistant to the higher concentrations of INH during the 2 subcultures, whereas FN remained about the same and ER seemed to increase very slightly in resistance. It must be remembered, however, that the 2nd test was performed on a pure culture, the inoculum of which was demonstrably greater than that of the original direct test. Strains FN and ER were also tested for their susceptibility to INSH. The results, as shown in Table I, indicate that they had increased their resistance to the same degree as they had to INH. Suspensions for inoculation were prepared by triturating the organisms in a mortar with the

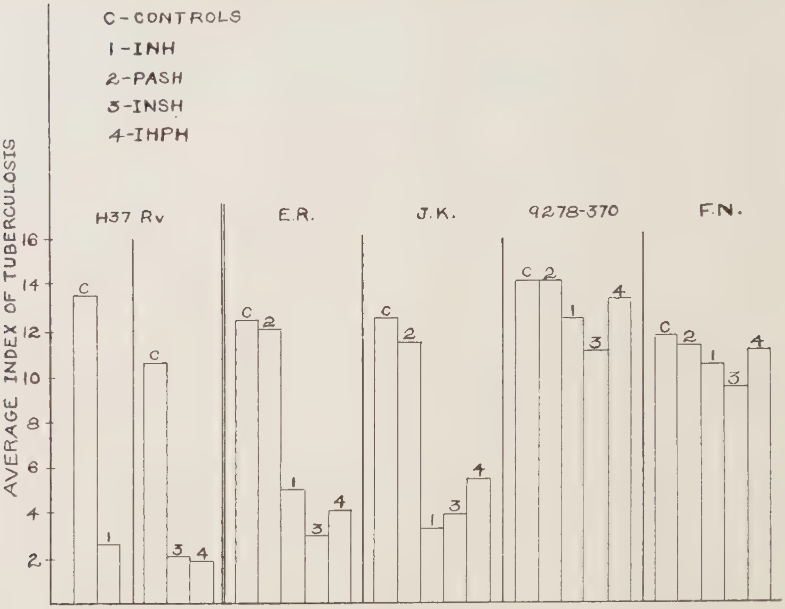

FIG. 1. Results of therapy with isoniazid (INH), N-isonicotinoyl-N′-(salicylidene) hydrazine (INSH), N-isonicotinoyl-N′-(2,2-dimethyl-3-hydroxypropylidene) hydrazine (IHPH), and PAS hydrazide (PASH) in guinea pigs infected subcutaneously with INH-sensitive and -resistant strains of tubercle bacilli. Avg index of tuberculosis based on amount of grossly visible disease in lungs, liver, spleen, and lymph nodes (each of these organs evaluated according to scale of 0 to 4+), with maximum total of 16.

In the first H37Rv experiment (reading from left to right), treatment was started 30 days after infection and the experiment terminated by sacrifice of all survivors 100 days after infection. In the 2nd H37Rv experiment, the corresponding figures were 18 and 60 days, and in the main experiment with the INH-resistant strains, 22 and 84 days.

aid of a pestle, using physiological saline as the diluent. Each guinea pig was inoculated subcutaneously in the inguinal region with approximately 0.1 mg dry weight. Treatment was delayed until 22 days after infection, at which time the 25 animals in each group were further divided into subgroups of 5; untreated controls, and those receiving each of the 4 drugs. The dosages of the drugs were as follows: INH‡, 10 mg, intramuscularly, once daily; INSH, 50 mg, by mouth, once daily; IHPH and PASH, 30 mg, by mouth, once daily. For the latter 3 drugs, this had previously been determined to be the maximum tolerated dosage for prolonged treatment. The oral doses were given in gelatin capsules. All survivors were killed to terminate the experiment 84 days after infection. Comparison of the various regimens was made on the basis of the amount of gross tuberculous disease in the lungs, liver, spleen, and lymph nodes.

Results. The results of this experiment are presented in Fig. 1. For comparison there are also shown the results of 2 similar experiments involving H37Rv-infected guinea pigs.

It may be seen that INH, INSH, and IHPH were about equal in their ability to suppress an experimental infection produced by the sensitive H37Rv strain. PASH was not tested in guinea pigs infected with a sensitive strain, but it produced no significant beneficial effect in those infected with any of the INH-resistant cultures. The animals infected with the 2 strains showing the least *in vitro* INH resistance, ER and JK, demonstrated a fairly good therapeutic result with INH and either of its 2 derivatives, while those infected with the 2 more resistant strains showed very little response to treatment with any of these drugs. In the latter 2 groups, treatment with INSH seemed to provide a slightly greater protection than either of the other 2 drugs, but the number of animals in each group was too small to draw any definite conclusions. In the 2 groups that responded fairly well to treatment, one (ER) showed slightly better results with INSH and the other with INH, but again the differences were relatively small. The result of therapy in the guinea pigs infected with strain ER, which contained the smallest number of INH-resistant mutants, was not as good as that obtained with the same drugs in infections produced by the sensitive H37Rv strain.

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‡ Supplied by Chas. Pfizer & Co., Brooklyn, N. Y.

PASH in Mouse Tuberculosis. This drug was evaluated in a group of C57 black mice infected intravenously with the H37Rv strain and treated immediately. PASH was dissolved in the drinking water so that each mouse received approximately 500 mg/kg/day. The experiment was terminated after 29 days. There was no difference between the treated and control groups as far as mortality and survival time were concerned, but the treated mice did show slightly less gross tuberculous lung disease and a slightly lower bacilli count in smears of the lung. Bavin *et al.*(19) reported that this drug had no activity in the mouse corneal test. Others(20,21) have reported slightly better results in guinea pigs.

INSH in Rabbit Tuberculosis. Nine rabbits of mixed breed weighing approximately 3.5 kg each, previously vaccinated subcutaneously with BCG to increase their native host resistance, were infected intravenously with 0.01 mg, dry weight, of the virulent bovine strain, 4228-4. This strain was susceptible *in vitro* to 0.1 μg of INSH per ml. The course of the disease was followed by serial chest roentgenograms according to the technique previously described(22). The 6 untreated rabbits all died of tuberculosis in an average time of 90 days. Three were treated with INSH starting 48 days after infection, when their x-rays showed advanced miliary pulmonary lesions. The drug was given by mouth in 50 mg capsules, the dosage being 100 mg twice daily (57 mg/kg/day) for 2 months. It was then reduced to 100 mg once daily because of weight loss, and continued at this level for the total treatment period of 6 months. During this time the lung lesions showed a progressive roentgenographic clearing approximately the same as that seen in similarly infected rabbits treated with INH in previous experiments (22). One animal died of a secondary infection after 113 days of treatment. At autopsy, scattered small lesions were still present in the lungs, from which tubercle bacilli susceptible to both INH and INSH were recovered in small numbers. The other 2 rabbits both showed a sudden relapse of pulmonary disease 4 months after the discontinuance of treatment.

Discussion. In the test tube it appeared that INSH retained a slight degree of activity against the INH-resistant H37Rv strain, and it was hoped that this would also be evident *in vivo.* Three of the 4 groups of guinea pigs infected with INH-resistant organisms responded slightly better to INSH than to INH or IHPH, but in the 4th group the best result was obtained with INH. Because of the small number of animals in each group and the slight differences noted, however, no significance can be attached to these observations. Clinical trials with INSH are now under way in Veterans Administration Hospitals in this country(23), as well as in other places. Additional evidence of the similarity between PASH and PAS is the report that the tuberculostatic activity of the former drug is antagonized by p-aminobenzoic acid(24).

The results of INH treatment in the guinea pigs infected with drug-resistant tubercle bacilli are of interest in view of the uncertainty as to what degree of *in vitro* resistance is significant clinically. The response of the animals infected with the strain of least resistance, ER with only 0.05% of the population able to grow in 1 μg/ml, was not as complete as the response in the H37Rv-infected groups. The animals infected with strain JK, which contained a much higher percentage of individual organisms resistant to 1 μg/ml, responded to therapy just as well as those in the ER group. Non-responsive infections were produced by the 2 strains containing a significant percentage of organisms able to grow in 5 μg/ml. These results are based on dosage of approximately 15 mg of INH/kg/day. They may be compared with those of Mitchison and co-workers(7,8) who found no response to INH treatment in guinea pigs infected with strains able to grow on 1 but not on 5 μg/ml with doses of 4 or 20 mg/kg/day.

Reginster(25) also described no response to INH treatment in animals infected with a strain resistant to 1.0 μg/ml.

Summary. 1. Two isoniazid derivatives, N-isonicotinoyl - N′ - (salicylidene) hydrazine (INSH) and N-isonicotinoyl-N′-(2, 2-dimethyl-3-hydroxypropylidene) hydrazine (IHPH), were found to have an *in vitro* tuberculostatic effect similar to that of INH but both exhibited cross resistance with the parent compound. H37Rv tubercle bacilli were easily made resistant to INSH by serial passage in drug-containing liquid medium, after which the strain was found to have lost its former virulence for guinea pigs. Resistance was much slower to appear, however, in medium containing a combination of INH plus INSH. 2. P-amino-salicylic acid hydrazide (PASH) showed about the same *in vitro* effect as, and cross resistance with, PAS, but there was no cross resistance with INH. Tubercle bacilli could easily be made resistant to PASH by serial passage in drug-containing medium. This compound demonstrated very little beneficial effect in mice infected intravenously with the H37Rv strain. 3. INSH produced therapeutic results similar to those seen with INH in experimental tuberculosis of rabbits infected intravenously with a virulent bovine strain. 4. Guinea pigs were infected subcutaneously with 4 INH-resistant strains of tubercle bacilli; 3 were human strains isolated from patients treated with the drug, and the 4th, a bovine strain isolated from a rabbit treated with INH. Treatment with INH, INSH, or IHPH produced good results in the animals infected with the 2 strains showing least *in vitro* resistance, but very little beneficial effect in those infected with the 2 strains containing at least 1.0% of individual organisms resistant to 5 μg/ml. There was no significant difference in the results obtained in any of the 4 groups with INH or either of its 2 derivatives. In none of the groups was treatment with PASH beneficial. 5. INSH and IHPH are similar in effect to INH in experimental tuberculosis produced by INH-sensitive and -resistant tubercle bacilli.

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Technic of Repeated Partial Hepatectomy in the Rat.* (21349)

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Following partial hepatectomy rapid regeneration occurs in the remaining lobes. In the rat the usual procedure is to excise the left lateral and median lobes, representing approximately 70% of the total liver. The history of earlier work has been given by Fishback(1) and by Brues, Druey, and Brues(2). We have developed procedures of surgery and postoperative care which permit removal of any part of rat liver, up to 80% of the total, and permit repeated partial removal of liver.

Technic. The following instruments were used: one pair of Halstead mosquito, 5-inch hemostatic forceps; one pair of iris scissors, straight, with sharp points; one pair Pley capsule forceps; one pair Prince trachoma forceps; one curved suture needle for muscle and skin; one Carrell arterial needle, taper point, straight, 20 mm; one aspirating pipette having 3 mm outside diameter and 25 mm length tip. The pipette is fitted into a 35-ml suction trap (glass vial). Negative pressure is created by a water pump operated by a foot pedal. Size 40 linen thread is used for ligatures on lobes of liver. Surgical silk is used to close skin and muscle. A fine thread of silk is used to close the capsule of liver. The principal conditions for the success of any surgery upon liver are the maintenance of hemostasis without blocking the arterial and venous blood and maintenance of biliary drainage. The experimenter should familiarize himself with the location of the vessels of each lobe. Any operative or postoperative deaths should be regarded as due to mishaps or errors of judgment subject to correction. Rats of any age can be used but we select animals of about 200 g weight. Ether anesthesia is preferred. Since the liver is the principal site of catabolism of barbiturates the partially hepatectomized rat does not recover readily from barbiturate anesthesia. Clean but not sterile technic is necessary. The first operation is done through a ventral midline incision extending posteriorly from the xiphoid. The incision should not be longer than is necessary to permit the delivery of liver. A tube of approximately one inch diameter is placed transversely under the back of the animal to aid in the extrusion of liver and intestines. Very gentle handling of the liver by the fingers is necessary. The surface of liver must not be broken or bruised by manipulation.

First stage of a series of partial hepatectomies. 1. *Right lateral lobe.* The head of the animal is to the experimenter's left. The right lateral lobe only is extruded. The anterior lobule is clamped and tied near its base and then excised. Nubbins of the lobule are left to be dealt with at the third step. The long tapering posterior lobule is lifted gently by the fingers of the left hand, clamped, ligated and excised. 2. *Caudate lobe.* The animal is turned with the head to the experimenter's right. The median and left lateral lobes are delivered so that they lie over the chest. The stomach, spleen and pancreas are delivered laterally, away from the experimenter. The mesentery is opened over the base of the caudate lobe near the oesophagus, the ligamentous attachments of the lobe are cut and the anterior and posterior lobules are gently pressed through the opening. The lobe is clamped and ligated at its base and excised. 3. *Liver around vena cava with remnants of right lateral and caudate lobes.* The animal is turned with its head to the experimenter's left. The median and left lateral lobes remain extruded over the chest and the stomach is delivered laterally, away from the experimenter. A Prince trachoma forceps is held in the left hand and applied to the vena cava so that the oval rings occlude the vena cava on each side of the remaining liver. The stumps of liver and the liver remaining around the vena cava are rapidly removed by negative pressure through the pipette. As quickly as this stage is completed the stomach and intestines are re-

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placed in the abdominal cavity so that their pressure aids in stopping hemorrhage from the small broken vessels. 4. *Left lateral lobe.* The rat is placed with its head towards the operator. The left lateral and the median lobes are extruded but the gut remains *in situ.* The left lateral lobe is clamped, ligated at its base and excised. The remaining nubbins are removed by aspiration. The remaining median lobe is replaced in the abdominal cavity and the muscle and skin are closed separately by continuous silk sutures. The order of the removal of lobes can be changed. Either the right lateral or the left lateral lobe may be left instead of the median lobe. The caudate lobe will not support life if all other parts of the liver are removed in one stage. The excised liver can be weighed. Aspirated liver is collected for weighing in a tared glass vial which serves as a suction trap.

Subsequent steps in series of partial hepatectomies. At least 2 weeks should lapse between operations. Incisions are now made to the left or right of the ventral midline. The median lobe, stomach and spleen are extruded. Using the arterial needle a fine silk thread is anchored in the capsule of liver near its caudal edge. If the capsule has become heavily fibrous at any point in this area it is advantageous to place the ligature there. At the ligature a small slit is made in the capsule to permit the tip of the pipette to be pushed into the liver. Holding the base of the lobe between the thumb and forefingers of the left hand will block the flow of blood. Liver substance is then aspirated. Not more than 40% of the liver substance should be removed. Some free blood is drawn with liver. This can be minimized by keeping the tip of the pipette against liver substance and by blocking the flow of blood into the cavity as indicated above. When the pipette is removed, pressure on the base of the liver is continued until the capsule is closed by continuous sutures with the arterial needle. Great care must be exercised to avoid rupture of the capsule and to avoid damage to the major vessels and the bile duct at the base of the lobe. The cavity left by removal of liver substance quickly fills with blood. Either the caudal tip or the left central lobule of the median lobe can be excised if the experimenter wishes to do so. Silk ligatures (interrupted) are placed in the liver to control hemorrhage. This procedure cannot be done more than twice. Aspiration of liver substance can be done every 21 days, or a little more frequently if desired. The capsule of the liver tends to thicken. Adhesions occur but should not prevent the ready extrusion of the lobe through the incision or visualization of the operative area.

Postoperative care. Extensive hemorrhage should not occur. If it does or if hemorrhagic shock is suspected, the animal can be given a direct transfusion. We connect the abdominal artery of the donor to the jugular vein of the recipient. All rats are given 20 ml of a 5% glucose solution by subcutaneous injection immediately after the operation and are given 10% sucrose solution to drink for at least 3 days. All rats receive 5 mg of streptomycin sulfate and 5000 units of procaine penicillin G by subcutaneous injection immediately following operation and each 24 hours for 7 days postoperatively. Each rat is separated in an individual cage for 7 days postoperatively, longer if healing of the skin is not complete by that time.

General observations. A number of other investigators have done as many as 3 extensive partial hepatectomies on the same animal. By use of the procedures described herein we have performed as many as 8 operations upon 6 young male rats having initial weights of approximately 225 g. The operations were performed every 21 days. An average total of approximately 40 g of liver per rat was removed during this period. At the end of this time the average body-weight was 474 g with a range of 422-512. The average weight of restored liver 3 weeks following the last operation was 11.5 g with a range of 8.7-13.3. Grossly, the liver was of normal color and was solid with almost no scar tissue. The vena cava of each liver was cavernous. There were many vessels branching over the surface of the restored livers.

Following extensive partial hepatectomy morphologic and functional recovery is incomplete at 21 days although the liver regains normal weight. The animal can survive the removal of up to 80% of liver at the first stage but the regenerated liver can never again withstand a single stage loss of this mass of tissue.

Signs of hepatic insufficiency are seen following partial hepatectomy, especially after 2 or more operations. These animals are prone to develop hypoglycemia. The natural resistance of the rat to infection is lost following extensive removal of liver, especially after 2 or more operations. Generalized fulminating infections develop and bring most of these animals to death even when rigid asepsis is maintained during surgery. This can be completely controlled by the use of antibiotics as indicated above.

After 2 or more operations most of the rats bite at their wounds and those of other rats and will disembowel themselves and their fellows if permitted to do so; hence the isolation of each rat in an activity restriction cage for one week or until healing of the wound is complete. We do not understand the basis for this behavior of the rat but it may relate to the unpleasant cutaneous sensations which develop in some patients with hepatic insufficiency.

Summary. Details of a technic for repeated partial hepatectomy in the rat are presented. Up to 80% of the liver can be excised and aspirated at the first stage. Any one of the 3 principal lobes (right or left lateral or the median) will support life after removal of all other remaining hepatic tissue. The caudate lobe will not support life if all other parts of liver are removed at the first stage. Following the restoration of liver additional partial removal of liver can be done by excision at the second and third stages. At all later stages the method of removing liver is that of aspiration. Careful attention to prevention of hypoglycemia, hemorrhage and infection insures uniform success of the procedure.

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Antigenically Modified Red Cells in Chickens Infected with Newcastle Disease. (21350)

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The antigenic changes produced in erythrocytes following adsorption and elution of certain hemagglutinating viruses have been considered in several recent investigations (1-5). Burnet and Anderson(3) demonstrated the appearance of new antigenic material on erythrocytes following treatment with viruses or *Vibrio cholerae* filtrates. They postulated that alteration of red cells by these agents *in vivo* could result in autoimmunization but were unable to demonstrate antibody for *in vitro* modified cells following the injection of viruses or RDE into guinea pigs. Karzon and Bang(6) detected a viremia followed by the appearance of agglutinins for virus treated red cells in chickens experimentally infected with Newcastle disease virus (NDV). Also, agglutinins and erythrocytes with trypsinized cell specificity have been demonstrated in acquired hemolytic anemia patients(7,8). In this laboratory we have shown the antigenic relationship among human red cells treated with NDV, influenza virus, and trypsin, and were able to obtain antiserum specific for NDV treated cells(5). Preliminary observations with this antiserum indicated the presence of erythrocytes with this specific alteration in chickens experimentally infected with NDV.

The present report deals with the preparation and use of a similar antiserum for a study of the occurrence of modified erythrocytes in chickens infected with NDV and the relationship of such cells to the viremia and to the presence of hemagglutinin inhibiting antibody and antibody for modified cells.

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Materials and methods. Viruses. Allantoic fluid containing influenza virus (PR8 strain) with a hemagglutinin titer of 4096 and an egg infectivity titer of $10^{-6.2}$ was used. The hemagglutinin titer of the Newcastle disease virus employed was 2048 and the egg infectivity titer was 10^{-11}. *Erythrocytes.* Normal human erythrocytes, O RH$^+$, and normal chicken red cells were washed 3 times in phosphate buffered saline (pH 7.4) before use. Virus treatments were performed by incubating 1 volume of washed packed red cells with 9 volumes of undiluted virus for 2-4 hours at 37°C. After treatment, erythrocytes were washed 3 times in buffered saline before use in immunizations, absorptions, and titrations. *Preparation and absorption of antisera.* Antiserum to erythrocytes were prepared by immunizing rabbits with normal, NDV and PR8 treated chicken red blood cells. The animals received 6 injections of 1.0 ml amounts of a 5% suspension of erythrocytes at 3 day intervals. Two weeks after the final injection the rabbits were bled and the sera collected, inactivated at 56°C for 30 minutes, and frozen until used. Antisera to normal and treated chicken red blood cells were absorbed with homologous and heterologous cells by incubation at room temperature for periods of 10 minutes and 30 minutes with equal parts of serum and packed cells. Subsequent absorptions were performed with a serum to cell ratio of 2:1 for periods of 1 hour. Virus antisera were obtained from rabbits 1 week after the last of 3 weekly intraperitoneal injections of 1.0 ml amounts of concentrated virus. *Serum hemagglutinin and virus hemagglutination inhibition.* Doubling dilutions of test sera were prepared in 0.1 ml amounts of phosphate buffered saline. One-tenth ml of a 2% suspension of test red cells was added to each serum dilution and tubes were incubated for 30 minutes at 37°C. Tubes were then centrifuged at about 1,000 r.p.m. for 1 minute and read for the presence of macroscopic agglutination. Virus hemagglutination inhibition tests were carried out by preparing doubling dilutions of serum in 0.25 ml amounts of buffered saline and adding four hemagglutinating units of virus contained in 0.25 ml, and 0.5 ml of a 0.25% suspension of chicken red cells. The inhibition tests were incubated for 1 hour at room temperature and read on the basis of the pattern of sedimented cells. *Experimental Newcastle disease in chickens.* Groups of 4-6 week old White Rock chickens were infected intracardially, intraperitoneally, intranasally, and intramuscularly with either undiluted virus or dilutions of virus ranging from 10^{-1} to 10^{-8}. Also, natural infection was simulated by placing uninoculated birds in pens with infected chickens. Normal, segregated animals of the same lot were used as controls in all experiments. Test and control animals were bled by cardiac puncture at the beginning of each experiment and at intervals after infection. Determinations of the presence of modified erythrocytes were made by the use of monospecific serum which is further discussed in the result section of this paper. Serum samples were also collected from infected and control animals in order to test for agglutinin to NDV modified erythrocytes and hemagglutinin inhibiting antibody.

Results. Rabbit antisera to normal and virus treated chicken erythrocytes demonstrated agglutinins for homologous and heterologous cells. Hemagglutinins for virus treated red cells were present in antiserum to normal chicken erythrocytes, and unabsorbed antisera to virus treated red cells reacted strongly with normal cells. Absorptions of antisera to normal and virus treated chicken erythrocytes with normal chicken red cells removed hemagglutinins for normal cells and

TABLE I. Titrations of Antiserum to Normal Chicken Erythrocytes.

Anti-serum	Absorbed with	Test red cells*		
		NC	CPR8	CNDV
NC	—	8192	16384	16384
"	NC	0†	2	4
"	NC and CPR8	0	0	4
"	NC and CNDV	0	2	0

* NC—Normal chicken erythrocytes; CPR8—Chicken erythrocytes treated with influenza virus; CNDV—Chicken erythrocytes treated with Newcastle disease virus.

† No agglutination in a 1:2 dilution of serum.

TABLE II. Titrations of Antiserum to PR8 Treated Chicken Erythrocytes.

Antiserum	Absorbed with	Test red cells*		
		NC	CPR8	CNDV
CPR8	—	1024	4096	4096
”	NC	0†	32	64
”	NC and CPR8	0	0	32
”	NC and CNDV	0	8	0

* See Table I for legend.
† No agglutination in a 1:2 dilution of serum.

TABLE III. Titrations of Antiserum to NDV Treated Chicken Erythrocytes.

Antiserum	Absorbed with	Test red cells*		
		NC	CPR8	CNDV
CNDV	—	4096	8192	16384
”	NC	0†	16	512
”	NC and CPR8	0	0	512
”	NC and CNDV	0	16	0

* See Table I for legend.
† No agglutination in a 1:2 dilution of serum.

left agglutinins with specificities for virus treated erythrocytes (Tables I, II, III). Further absorptions with treated cells resulted in the separation of agglutinins with specificity for the individual virus treated erythrocytes.

Antigenic modification of red cells *in vivo* was detected by employing a monospecific antiserum obtained by the absorption of antiserum to chicken erythrocytes treated with NDV *in vitro* with normal and PR8 treated cells. The data in Table III show the original specificities present in this serum, and that after absorptions with heterologous cells, this serum agglutinated only erythrocytes previously treated with NDV. The absence of NDV hemagglutinin inhibiting antibody in the sera of the chickens before infection indicated no previous contact with this virus. Antigenically modified erythrocytes were detected in chickens infected by the intracardial, intraperitoneal and intranasal routes, as well as in animals which had been infected as a result of contact with diseased animals. Table IV presents a comparison of the time period in which modified red cells were detected in the chickens infected by the various routes. The numerical values refer to the titer of the test serum with the erythrocytes obtained at each time interval listed.

Modified red cells were detected in each of the chickens infected intracardially 4 hours after infection. Symptoms appeared in 24-48 hours with death occurring in 70% of the animals by the 5th day. Modified cells were demonstrable until death, or in the surviving animals for 11 days after infection. Egg inoculations using whole blood from the infected animals revealed that a viremia was present when first tested for at 24 hours, and persisted until the 5th day. Therefore, modified cells were demonstrable after disappearance of detectable virus from the circulation. The appearance of modified red cells, the onset of symptoms and time of death were delayed in chickens inoculated by other routes, but except for the group injected intraperitoneally, the altered red cells persisted for approximately the same time in all groups. Thus, cell modification in the group of chickens infected intraperitoneally was not detected until 12 hours after infection and persisted only through the 5th day, after which time it was no longer demonstrable in the survivors. Symptoms appeared somewhat later than in the previous group and approximately 50% of the animals died. Modified cells were detected 3 days

TABLE IV. Titers of Antiserum to NDV Treated Red Cells with Erythrocytes from NDV Infected Chickens.*

Time after infection	Routes of infection			
	Intracardial†	Intraperitoneal†	Intranasal‡	Natural§
4 hr	64	0	0	0
8	64	0	0	0
12	128	4	0	0
16	128	4	0	0
24	256	8	0	0
2 days	128	16	0	0
3	64	8	8	0
4	64	4	8	4
5	64	0	16	4
6	64	0	32	8
7	64	0	64	16
8	64	0	64	32
9	64	0	32	64
10	32	0	8	64
11	16	0	8	32
12	0	0	4	32
15	0	0	0	8
16	0	0	0	0
37	0	0	0	0

* Erythrocytes from test chickens before inoculation and from control animals were not agglutinated by the antiserum used.
† Avg titers obtained with 20 chickens.
‡ ” ” ” ” 10 ” .
§ ” ” ” ” 8 ” .

after intranasal inoculation of virus and persisted until the 12th day after infection. Death occurred in approximately 60% of these chickens between the 8th and 12th day of the disease. The simulated natural infection resulted in detectable cell modification as early as the 4th day of exposure and persisted through the 12th day.

As shown in Table IV maximum titers of the monospecific serum were obtained with red cells from bleedings 12 to 24 hours after the intracardial injection of the virus. These titers are approximately the same as those obtained using *in vitro* treated erythrocytes as test cells.

Repeated attempts to detect modified cells in chickens infected by intramuscular inoculation failed, although the typical disease usually followed. Subsequent tests on the serum of these animals showed significant amounts of agglutinins for modified cells, indicating the probability that undetected cell modification had occurred. Modified cells could not be demonstrated in immune chickens injected with large amounts of virus.

Modified cell agglutinins were tested for in the sera of the chickens infected by the different routes employing chicken erythrocytes treated with NDV *in vitro*. Agglutinins were present in all animals infected by the different routes, although, variations in titers were noted. Maximum titers of 1:64 were obtained between the 5th and 6th days of infection and agglutinins were still present at titers of 1:8 as long as 37 days after the initial infection. Highest titers were obtained by intracardial infection with the lowest observed in those animals receiving the virus intraperitoneally. Agglutinins were also found in the sera of the animals infected intramuscularly even though modified cells were not detected during the course of the infection.

Hemagglutinin inhibiting antibody was present on the 5th day after infection and reached a maximum in approximately 3 weeks, after which it gradually decreased in titer but was still present at the end of the test period.

Discussion. Antigenic modification of red cells during Newcastle disease in chickens is indicated by several facts. (1) Erythrocytes from infected chickens possess specificity similar to that of red cells treated with NDV *in vitro*. (2) Modified erythrocytes persist beyond the time that virus is detectable in the blood. (3) Hemagglutinins with specificity for red cells treated with NDV *in vitro* are produced.

Absorbed antisera to erythrocytes treated with NDV *in vitro* agglutinate red cells from infected chickens. Similarly, antiserum to cells modified *in vivo* reacts specifically with erythrocytes treated with NDV *in vitro*(9). Antiserum to NDV treated cells not only differentiate these cells from normal erythrocytes but also from red cells treated with the similarly acting mumps and influenza viruses(9).

Persistence of modified erythrocytes in NDV infected chickens beyond the time that detectable blood virus was present suggests that virus-red cell interaction takes place *in vivo* in a manner similar to that *in vitro*. Thus, the erythrocyte is altered antigenically *in vivo* by virus action rather than merely carrying the virus particle. This is substantiated further by the subsequent appearance of antibody for virus treated cells. This antibody can be separated from the virus hemagglutinin inhibiting antibody(5), and is produced at a rate different from antibody to the virus.

Cellular antibody in infected chickens may represent an "auto"-immunization by the modified erythrocytes; a phenomenon analogous to the production of antibody to homologous brain or kidney tissue(10,11) and one that has been suggested for the appearance of autoantibody in acquired hemolytic anemia patients(8). In this laboratory, autoimmunization of rabbits has been accomplished using trypsinized red cells and combination of antibody and erythrocytes *in vivo* has been demonstrated(12).

Perhaps the distribution of virus following intramuscular inoculation minimizes the opportunity for action on red cells to a degree not detectable with antiserum but sufficient to provide antigenic stimulation. Likewise, infected cells of other tissues undoubtedly are modified and may serve as antigen. Investigation of the diagnostic possibilities of these technics in other diseases in which virus exists in the circulation for some time is indicated. Experimental Newcastle disease in chickens also affords an opportunity for the investigation of the fate of modified erythrocytes and the possible role of "auto-antibody" in the process.

Summary. 1. Antisera specific for either NDV or influenza virus-treated chicken erythrocytes were prepared. 2. Virus modified red cells were detected in chickens infected with NDV by various routes of inoculation by use of the specific antiserum. 3. Hemagglutinins for NDV-treated chicken red cells were also detected in the serum of infected chickens.

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Chromatographic Investigation of Purines in Normal Human Urine.* (21351)

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Present knowledge of the urinary excretion of purines other than uric acid and the methyluric acids derives largely from the classical work of Krueger and Salomon(1). These investigators were able to isolate from 10,000 liters of urine the compounds xanthine, hypoxanthine, adenine and "epiguanine" (7-methylguanine) in amounts of 0.3-1 mg/liter. Paraxanthine (1, 7-dimethylxanthine), heteroxanthine" (7-methylxanthine) and 1-methylxanthine, obtained in somewhat greater amounts, were subsequently(2) shown to derive from dietary tea and coffee.

The present communication describes a new method for the separation and identification of non-uric acid purines which is suitable for the examination of individual urine specimens. Preliminary results in normal human subjects are reported.

Methods. In the procedure which follows, removal of the uric acid, urea and salts is accomplished by an ion exchange process, the purines are separated from the remaining creatinine and amino acids by precipitation as their silver nitrate salts, and the concentrate is separated by paper chromatography. One-tenth of a 24-hour specimen of urine, acidified to pH 2, is passed through 50 ml of Dowex 50—H^+. The column is washed with water and eluted with 200 ml of 1 *M* ammonium hydroxide. After concentration of the eluate *in vacuo* to 10 ml, sulfuric acid is added to pH 2, followed by 2 ml of 1 *M* silver nitrate. After several days, the resulting precipitate, sparingly washed with water, is extracted with hot 0.05 *M* hydrochloric acid. The extract is con-

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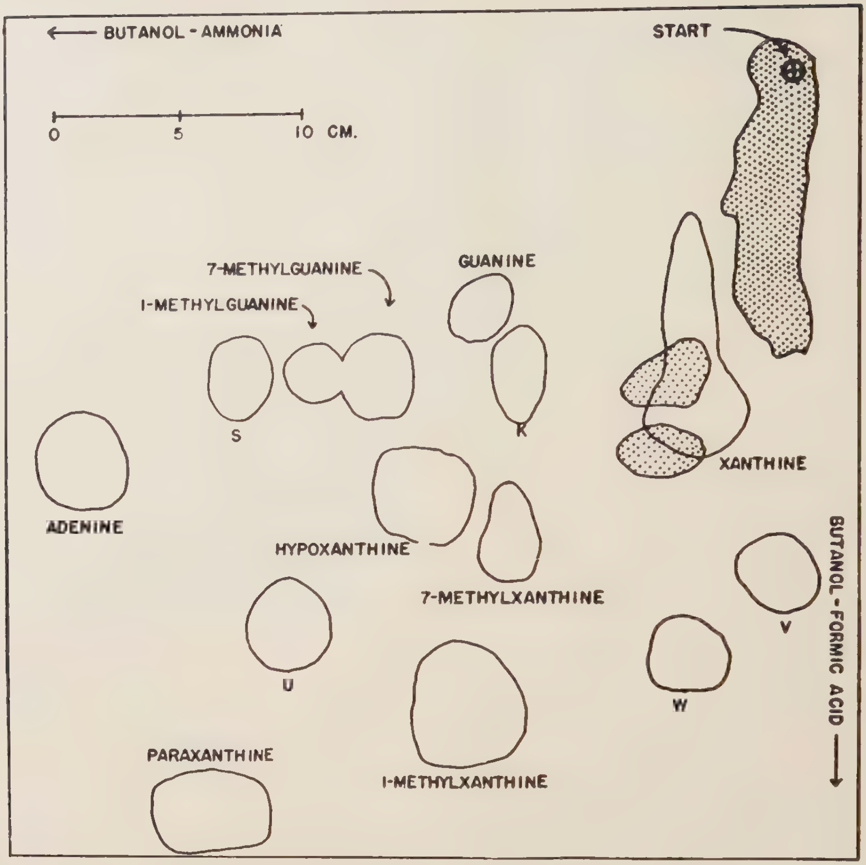

FIG. 1. Map of 2-dimensional chromatograms of urinary purine concentrates from normal subject on unrestricted diet, or on purine-free diet with caffeine supplement. Purine spots appear in ultraviolet light as dark spots on weakly fluorescent paper. Strongly fluorescent areas, also indicated, are shaded with dots. In the absence of caffeine, coffee, etc., spots for paraxanthine, 7-methylxanthine, and 1-methylxanthine are not observed.

centrated *in vacuo* to dryness, and the residue is at once redissolved in 0.80 ml of 1 *M* hydrochloric acid. The *concentrate* is applied in 3 successive 20 μl spots to a sheet of Whatman 3 MM filter paper, which is developed by downward irrigation as a 2-dimensional chromatogram. n-Butyl alcohol-0.6 *M* ammonium hydroxide, 6:1(3), is used in one direction for 36 hr. n-Butyl alcohol - 88% formic acid-water, 77:11:12(4), is used in the other direction for 8 or 14 hours. Purine spots are visualized in ultraviolet light, cut out of the paper, and eluted with water. The *eluates* are used for determination of ultraviolet spectra, using as blank correction values obtained with an identically treated sheet of paper to which no purine has been applied. Purines are identified by comparison of their mobilities on paper with the two solvents used, and of their spectra at pH 2 and pH 9 with those of authentic specimens.† The quantity of material recovered is determined from the absorption at one of the peaks. Where the substance is unidentified or its extinction coefficient is not known, the value $E_{max} = 0.7$ (1 cm, 10 mg/liter), an average value for many purines, is used. Two male subjects, one age 24, weight 88 kg, the other age 37, weight 93 kg, in apparent good health, were the source of speci-

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TABLE I. Spectral Constants and Mobility on Paper of Purines Isolated from Urine.

	R_F values			λmax and relative extinction of peaks	
Compound	From urine	Authentic	pH	Substance from urine	Authentic compound
Hypoxanthine	.13	.12	2.0	250 (1.00)	250 (1.00)
	.26	.26	9.0	256 (.97)	257 (1.00)
Xanthine	.03	.02	2.0	266 (1.00)	266 (1.00)
	.19	.20	9.0	240 (.90); 276 (.92)	240 (.88); 277 (.97)
7-Methylguanine	.15		0–2	250 (1.00); ca. 275 (.64)	250 (1.00); ca. 275 (.60)
	.12		6	248 (.50); 283 (.71)	245 (.62); 281 (.74)
			12–14	281 (.71)	282 (.73)
Adenine	.33	.32	2.0	262 (1.00)	264 (1.00)
	.26	.25	9.0	261 (.97)	262 (1.00)
1-Methylguanine	.17	.17	2.0	251 (1.00); ca. 275 (.62)	251 (1.00); ca. 275 (.69)
	.13	.12	9.0	249 (.80); 275 (.72)	249 (.90); 274 (.75)
Guanine	.12	.11	2.0	248 (1.00); ca. 275 (.58)	247 (1.00); ca. 275 (.64)
	.12	.12	9.0	248 (.71); 275 (.64)	245 (.83); 275 (.72)
1-Methylxanthine	.08	.07	2.0	267 (1.00)	267 (1.00)
	.44	.43	9.0	243 (.72); 276 (.89)	243 (.75); 277 (.86)
7-Methylxanthine	.06	.06	6.0	268 (1.00)	268 (1.00)
	.29	.29	9.0	283 (.70)	280 (.70)
Paraxanthine	.27	.25	6.0	269 (1.00)	267 (1.00)
	.53	.53	9.0	285 (.74)	282 (.69)

R_F values given are mobilities with butanol-ammonia and butanol-formic acid respectively, run on Whatman No. 1 filter paper by the descending technic, of materials eluted from 2-dimensional chromatograms, as compared with authentic specimens.

Values given for spectral constants of authentic purines are those from the literature for hypoxanthine(5), xanthine(5), 7-methylguanine(6), adenine(5), guanine(5), 7-methylxanthine (7), and paraxanthine(7). For 1-methylguanine and 1-methylxanthine, values are obtained in present work with purified synthetic specimens.

mens. After a period of unrestricted food intake, including tea and coffee, they were maintained on a low protein diet free of meat and known methylxanthine sources. At the end of 7 days, one of the subjects, while still on diet, was given 600 mg of caffeine daily. The 24-hour specimens of urine examined were taken before start of the diet, on the seventh day of diet, and on the second day of caffeine administration.

Results. The type of chromatogram obtained by the procedure described is shown in Fig. 1, where the names of the purines so far identified are also given. The evidence on which identifications are based is summarized in Table I. In the case of 7-methylguanine, a pure synthetic sample was not available, and identification is based on comparison of the spectrum obtained with that reported earlier for this compound (Fig. 2). The correspondence here of the spectrum from paper elution with that obtained with the crystalline compound is representative of the other compounds described. In agreement with older work, spots for paraxanthine, 7-methylxanthine, and 1-methylxanthine are not detected when coffee, tea and chocolate(2) are withheld from normal subjects, but appear when caffeine is added to the diet(8).

Representative quantitative data are given in Table II. No significant differences are observed between the excretion patterns of the 2 normal subjects examined.

Replicate experiments in general show a precision better than ±10%, except for the faintest spots which are difficult to localize. That recovery of the purines is considerably lower than this precision indicates is shown by recovery experiments with hypoxanthine, adenine, and guanine (85%, 63% and 70% recovery). The recovery of xanthine, which gives a smeared spot with the formic acid solvent (Fig. 1) at the levels obtained from urine, is lower (60%).

Discussion. Qualitatively, the present results confirm those of Krueger and Salomon

(1) in demonstrating the presence in normal human urine of the free purines hypoxanthine, adenine, xanthine and 7-methylguanine. Guanine and 1-methylguanine are now also shown to be present. Guanine has been reported as a urinary constituent by Pouchet(9), but the validity of the evidence for this finding was disputed by Krueger and Salomon(1). 1-Methylguanine has not previously been reported from biological sources. Five of the substances which appear on the chromatogram (Fig. 1) display absorption maxima in the region 240-300 mμ, and may be purines, but are as yet unidentified.

The levels of the non-uric acid purines were found to be as much as 10-fold higher than those previously reported(1). Nevertheless, the values here recorded are probably low, since the recovery of added known purines is not quantitative.

Whether the free purines are exogenous or, as appears more likely from the absence of any pronounced dietary effect (Table II), endogenous in origin is not yet established. If endogenous, the position of these compounds, especially the methylated purines, in human metabolism remains to be elucidated.

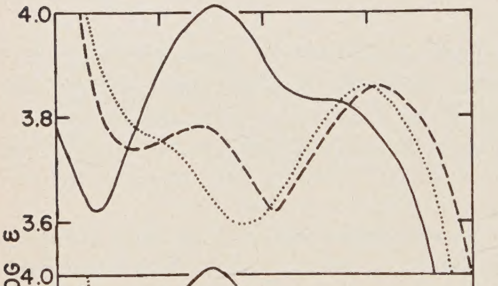

FIG. 2. Ultraviolet absorption spectrum of 7-methylguanine from urine (upper curves) and spectrum(6) after Gulland and Story (lower curves). Solid line, $N/20$ HCl; dotted line, $N/20$ NaOH; dashed line, neutral solution.

TABLE II. Normal Urinary Excretion of Purines (mg/Day).

Compound	Low protein diet	Caffeine added	Unrestricted diet
Hypoxanthine	10	10	8
Xanthine	4	5	7
Adenine	1.4	1.0	1.4
Guanine	.4	.4	.6
7-Methylguanine	4.5	5.9	6.3
1-Methylguanine	.5	n.r.	n.r.
Spot K	1.5	1.8	1.4
" S	.7	.4	.4
" U	.7	.4	.6
" V	1.2	1.4	1.5
" W	1.0	1.1	1.2
7-Methylxanthine	abs.	19	pres.
1-Methylxanthine	"	52	"
Paraxanthine	"	17	12

abs. = not detected; pres. = presence noted, amount not measured; n.r. = not resolved, measured as 7-methylguanine.

Summary. A method has been developed by which individual variations in urinary excretion of purines may be investigated on a qualitative and semi-quantitative basis. The method employs an ion exchange step to remove uric acid, and separates by paper chromatography the purines precipitated by silver nitrate. Two normal subjects on purine-free diet excreted a total of more than 30 mg/day of non-uric acid purine derivatives. Those identified, in order of diminishing abundance, are hypoxanthine, xanthine, 7-methylguanine, adenine, 1-methylguanine and guanine.

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Effect of SKF Compound 525A on Excretion of Pentothal-S^{35} in Mice.* (21352)

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Barbiturate-induced sleeping time can be significantly prolonged in experimental animals by a number of chemical compounds (1,2). This phenomenon has no application which is immediately practical, but may be employed as a useful tool for the study of mechanisms involved in central nervous depression by the barbiturates. SKF compound 525A[†] has been recently reported to prolong hexobarbital-induced sleeping time in mice(3). We have extended consideration of this compound's action to Pentothal-induced anesthesia and the excretion of S^{35}-labeled Pentothal in mice; some correlation between the retention of S^{35} and the prolongation of sleeping in SKF-treated animals will be shown to obtain under the conditions of these experiments.

Methods. The determination of SKF-525A effect on sleeping time (S.T.) induced by Pentothal was carried out at two dose levels, as preliminary experiments showed that some relation existed between the dose of SKF and the response in the Pentothal-treated animal. White mice (male) weighing 18-20 g were administered 50 mg/kg (10 animals) and 75 mg/kg (10 animals) of SKF-525A (intraperitoneally) 40 minutes(3) before intravenous injection of 70 mg/kg of Pentothal. Control animals received only Pentothal, intravenously (20 animals). The ability to right was used as the criterion of recovery.

Results of the determinations are presented:

Dose SKF (mg/kg)	Pentothal (mg/kg)	Mean S.T. treated (min.)	Mean S.T. control (min.)
50	70	78.5	30
75	70	147	34

Prolongation of S.T. by SKF-525A

50 mg/kg	75 mg/kg
2.6	4.3

These data indicate that the prolongation of Pentothal sleeping time is dependent on the dose of SKF-525A administered.

The prolongation of Pentothal sleeping time by SKF-525A suggested that one of the factors involved might be barbiturate retention. As the S^{35}-labeled compound was available,[‡] it was possible to study these suspected changes using the tracer technic. Male white mice (18-20 g) were given intravenous Pentothal-S^{35} equivalent to 1,113,650 counts per 20 g body weight (2.1 x 10^6 disintegrations per minute at counter efficiency of 53%). This dose was not sufficient to cause symptoms of barbiturate intoxication. The series of SKF-treated animals was premedicated with 75 mg/kg (intraperitoneal injection) 40 minutes before the administration of Pentothal. All animals (5 in each group) were placed in glass metabolism cages and urine collected hourly. Suitable aliquots of urine were plated on copper discs and assayed for radioactivity in open-window, gas-flow Geiger counters. The activity recovered in each hour's sample was expressed as the percent injected dose and the mean percent taken for each group of 5 animals per hour. The data are presented in the following table:

Hr	Treated (SKF)	Control
1	0	13.455
2	4.746	5.651
3	6.191	3.859
4	11.228	3.680
5	4.493	0.968
6	1.364	1.178
7	3.085	1.293
Mean % radioactivity recovered in 7 hr	31.1%	30.08%

As the changes sought were observed within a few hours after injection the experiment was terminated at the end of 7 hours, for S^{35} excretion is considerably protracted in Pentothal-treated mice and it was felt that inclusion of

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[*] This work was supported by a grant from the Atomic Energy Commission and the Wallace C. and Clara Abbott Fund.

[†] Kindly supplied by Smith, Kline & French Laboratories.

[‡] Obtained from Abbott Laboratories, North Chicago, Ill.

data beyond this time would be irrelevant to the problem at hand. Measurements of S^{35} excreted from 7 to 18 hours substantiated this contention; the pattern was quantitatively similar for both SKF-treated and control animals.

The following observations can be made on the basis of the results obtained: 1. The excretion of Pentothal is significantly delayed by pretreatment with SKF-525A. 2. The excretion of Pentothal by the treated series appears to be delayed sufficiently to account partially for the prolongation of sleeping time. Some interference with excretion and/or metabolism of the drug may be suspected. 3. The mean percentages of injected radioactivity excreted in 7 hours by both groups are essentially identical; the delay in excretion attributable to SKF is offset by gradually increasing urinary S^{35} up to 4 hours, while control S^{35} decreases simultaneously. 4. The delayed S^{35} excretion in animals treated with SKF indicates the possibility of differences in distribution of the drug at a given time, either with respect to quantity or organs involved. This problem is at present under investigation and preliminary findings indicate a significant alteration in blood, brain and liver S^{35} levels in the SKF-treated animals. Effects on the metabolism of Pentothal, if any, have not been investigated.

The mechanism by which SKF-525A influences barbiturate sleeping time cannot be as yet clearly defined; however, some preliminary studies(4) have made the approach to this problem apparent, for it has been found that doses of the SKF compound in rats radically alter the partition of glucose into brain, and on a time basis presumably decrease the turnover of this compound to a fraction of the normal rate(4). These observations are being extended to the action of SKF-525A in conjunction with other barbiturates and morphine in hopes that more information relative to the effects of these depressants on nervous tissue can be obtained.

Summary. Administration of SKF Compound 525A to mice (50 mg/kg, 75 mg/kg) prolongs significantly Pentothal-induced sleeping time, the duration of which is dependent on the dose of SKF-525A. The excretion of tracer amounts of Pentothal-S^{35} has been shown to be delayed by pretreatment with SKF-525A. Over a 7 hour period control animals excreted the maximum percentage of the injected dose (13.455%) within one hour, while treated animals retained S^{35} until the fourth hour after injection (11.228%). At the end of the 7 hour observation period, 31.1 and 30.08% of the injected doses were recovered from treated and control series, respectively. These observations are being extended to other barbiturates and the possible mechanism of action of SKF-525A.

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